Functional and Physical Interactions of 14-3-3 Proteins with Novel Targets upon Replication Stress in *S. cerevisiae*

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2. ABSTRACT

The integrity of DNA replication forks is important for cell viability. In replication checkpoint-deficient budding yeast, disturbances caused by DNA damaging agents possibly result in fork collapse or DNA breaks/rearrangements. Previous studies from our laboratory indicated that 14-3-3-deficient yeast cells cannot restart replication forks in response to hydroxyurea (HU) treatment. The data also indicated that in these cells ssDNA gaps accumulate behind the fork in an Exo1-dependent manner. However, while deletion of *EXO1* rescued the accumulation of ssDNA gaps, it was unable to rescue HU sensitivity and the slow fork progression/restart defect of 14-3-3-deficient cells. These studies highlighted the fact that additional unknown targets of 14-3-3 proteins contribute to promote fork progression, stability and restart. From a list of established 14-3-3-interacting proteins selected *in silico* on the base of their involvement in DNA transactions, we attempted to identify factors that suppress fork restart upon HU-release in 14-3-3-deficient cells. We obtained evidence that Dpb3, one of the two accessory subunits of Pol ε, physically interacts with yeast 14-3-3 (Bmh1) *in vivo* and that deletion of *DPB3* partially suppresses the HU sensitivity of 14-3-3-deficient cells. Extension of our analysis showed that *DPB3* deletion causes partial rescue of fork restart defects as well as cell cycle defects of the 14-3-3-deficient strain. However, further analysis with 2D gel electrophoresis revealed faster fork progression in *dpb3*Δ cells under conditions of low dNTPs, arguing that *DPB3* deletion alone is sufficient to accelerate replication forks and, overall, S phase progression. Contrary to what observed with *DPB3*, deletion of the gene coding for the other accessory subunit of Polε, *DPB4,* did not affect the slow HU recovery phenotype of 14-3-3-deficient cells. Similarly, our analysis ruled out the possibility of an involvement of translesion synthesis (TLS) polymerases in the fork acceleration phenotype of *dpb3*Δ cells. Last conducted experiments focused on understanding the molecular mechanism by which *DPB3* deletion affects the fork progression, the extent of checkpoint activation and the level of enriched ssDNA gaps upon HU treatment. Overall, our studies on physical and functional interactions between Bmh1 and an accessory subunit of Pol ε reveal a novel function for Dpb3 upon replication stress in *S. cerevisiae.* This study will help expanding our knowledge on pathways controlling processive DNA synthesis and its link to genomic stability.

3. ZUSAMMENFASSUNG

Die Unversehrtheit der DNS-Replikationsgabeln ist von grosser Bedeutung für die Lebensfähigkeit von Zellen. In *Saccharomyces cerevisiae* ohne Replikations-Checkpoint können Störungen, welche durch DNS-schädigende Substanzen hervorgerufen wurden, zu Replikationsgabel-Zusammenbruch oder DNS-Brüchen/-Umgruppierungen führen. Frühere Studien aus unserem Laboratorium zeigten, dass Hefezellen, die kein 14-3-3 Protein besitzen, die Replikationsgabeln nach einer Hydroxurea (HU)-Bahandlung nicht wieder neu starten können. Die Daten zeigten ausserdem, dass sich in diesen Hefezellen Einzelstrang-DNS Lücken hinter der Replikationsgabel ansammelten, dies in Abhängigkeit von Exo1. Dennoch, während die Deletion von *EXO1* der Ansammlung der Einzelstrang-DNS Lücken entgegenwirkte, konnte sie die Empfindlichkeit der Zellen gegenüber HU und die langsamen Replikationsgabel-Progression/-Neustart in den 14-3-3 defizienten Zellen nicht vorbeugen. Diese Studien deuteteten darauf hin, dass zusätzliche unbekannte Zielproteine von 14-3-3 Proteinen zum Vorantreiben der Replikationsgabel-Progression, -Stabiliät und -Neustart beitragen. Wir haben in silico Proteine aufgrund ihrer Einbindung in DNS-Transaktionen ausgesucht und daraus eine Liste von denen aufgestellt, die mit 14-3-3 Proteinen interagieren. Aus diese Liste haben wir versucht diejenigen Proteine zu indentifizieren, die den Replikationsgabel-Neustart nach HU-Behandlung unterdrücken, wenn 14-3-3 in den Zellen abwesend ist. Wir fanden heraus, dass Dpb3, eine von den beiden zusätzlichen Untereinheiten der Polymerase ε, physisch und in vivo mit dem Hefeprotein 14-3-3 (Bmh1) interagiert und, dass die Deletion von *DPB3* teilweise die HU-Empfindlichkeit der 14-3-3 defizienten Zellen unterdrückt. Weitere Analyse zeigte, dass *DPB3* Deletion zu einer partiellen Umkehr der Defekte des Replikationsgabel-Neustarts und auch des Zellzyklus in dem 14-3-3 defizienten Stamm führte. Jedoch wurde mit 2D Gelelektrophorese unter den Bedingungen mit niedriger dNTP-Menge eine schnellere Replikationsgabel-Progression in *dpb3Δ* Zellen sichtbar, was dafür sprechen würde, dass *DPB3* Deletion allein für das Beschleunigen der Replikationsgabeln, und der S-Phase Progression generell, ausreichend ist. Im Gegensatz zu den Beobachtungen mit *DPB3*, beeinflusste die Deletion des Gens, das für die zweite zusätzliche Untereinheit von Pol ε kodiert, *DPB4*, den Phänotyp der langsamen Erholung nach HU Behandlung in

14-3-3 defizienten Zellen nicht. Ebenso hat unsere Analyse die Möglichkeit der Einbindung der "translesion synthesis" (TLS) Polymerasen im Phänotyp der beschleunigten Replikationsgabeln in *dpb3Δ* Zellen ausgeschlossen. Weitere Experimente konzentrierten sich auf die Aufklärung des molekularen Mechanismus, durch den die *DPB3* Deletion die Replikationsgabel-Progression beeinflusst, den Umfang der Checkpoint-Aktivierung und der Menge der angereicherten Einzelstrang-DNS Lücken nach HU Behandlung. Insgesamt wird durch unsere Studien über die physischen und funktionellen Interaktionen zwischen Bmh1 und den zusätzlichen Untereinheiten von Pol ε eine neuartige Funktion von Dpb3 nach Replikationsstress in *S. cerevisiae* zugewiesen. Diese Studie wird dabei helfen die Kenntnisse über Signalwege, die die prozessive DNA Synthese kontrollieren, und deren Link zu genomischer Stabilität, zu erweitern.

4. INTRODUCTION

4.1 The Eukaryotic Cell Cycle

The cell cycle is the orderly sequence of events performed by cell in which it duplicates its contents and then divides in two. This cycle of duplication and division is the fundamental mechanism for passing on its genetic information to the next generation of cells (Hartwell, Culotti et al. 1974). Thus the most basic function of the cell cycle is to duplicate accurately the vast amount of DNA in the chromosomes and then segregate the copies precisely into two genetically identical daughter cells (Hartwell, Culotti et al. 1974). These events define two major phases of the cell cycle: S phase (S for synthesis) in which DNA duplication occurs and M phase (M for mitosis) in which chromosome segregation and cell division occur. In a typical mammalian cell, S phase requires 10-12 hours and M phase occupies only less than an hour (Nurse, Thuriaux et al. 1976). But, most cells require much more time to grow and double their mass of cytoplasmic content during the cell cycle.

In order to allow more time for growth, extra gap phases are inserted – G_1 phase characterized by cell growth and synthesis of components for progression through cell cycle and G_2 phase after S phase (Tessema, Lehmann et al. 2004). These two gap phases not only provide time to allow cell growth but also time for the cell to monitor both internal and external environment. If conditions are unfavorable, cells delay progress through G_1 and in some cases they enter a special resting state known as G_0 in which cells do not proliferate at all (Nasmyth 1996).

The time required for cells completing one entire cell cycle is highly variable among different cell types. However, the basic organization of the cycle and its control system are essentially the same in all eukaryotic cells (Nasmyth 1996).

4.1.1 Cell cycle control system

Due to its requirement for cell growth and viability, cell cycle is the most fundamental process, which needs to be tightly regulated. The main regulators of the cell cycle are the cyclins, which peak in quantity during particular phases of the cell

cycle and their partners the cyclin-dependent kinases (Cdks). Those serine/threonine kinases form a complex with their cyclin partners and phosphorylate cell cycle components which, in turn, trigger cell cycle events (Koch and Nasmyth 1994) (McDonald and El-Deiry 2000). Cyclin abundance is regulated by protein synthesis and degradation, whereas the activity of Cdks is regulated to a large degree by the presence of different cyclins. There are four major classes of cyclins, based primarily on the timing of their expression and their function in the cell cycle:

i. G_1 cyclins contribute to the control of cell cycle entry in response to extracellular factors.
ii. G_1/S cyclins bind Cdks in late G_1 initiating the processes leading to DNA replication.
iii. S cyclins bind Cdks during S phase and are necessary for stimulating DNA replication.
iv. M cyclins promote the events of mitotic spindle assembly and alignment of sister chromatid pairs.

In yeast cells, a single Cdk protein (Cdc28) binds all classes of cyclins and drives all the cell cycle events by changing cyclin partners at different stages of the cycle. In vertebrates, by contrast, there are four major Cdks (Morgan 2007) (Alberts 2002).

CYCLIN–CDK COMPLEX	VERTEBRATES		BUDDING YEAST	
	CYCLIN	CDK PARTNER	CYCLIN	CDK PARTNER
G_1-Cdk	cyclin D*	Cdk4, Cdk6	Cln3	Cdk1
G_1/S-Cdk	cyclin E	Cdk2	Cln1, 2	Cdk1
S-Cdk	cyclin A	Cdk2, Cdk1	Clb5, 6	Cdk1
M-Cdk	cyclin B	Cdk1	Clb1, 2, 3, 4	Cdk1

Among all cyclins, G_1 cyclins are not essential and thus not required in all eukaryotic cells (Stillman 1996) (Ewen, Sluss et al. 1993). A simplified view of the cell cycle control system in budding yeast with different cyclins and entry points is shown in figure 1.

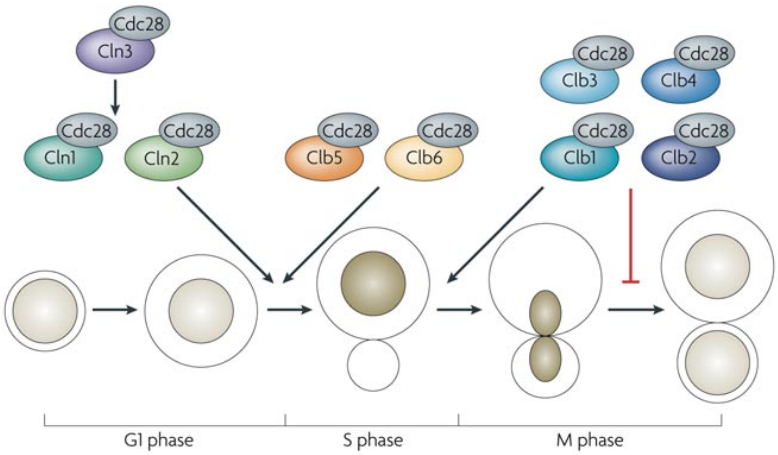


Figure 1: How different cyclin-Cdk complexes act throughout the cell cycle

Modified after (Bloom and Cross 2007)

4.1.2 Cell cycle control in *Saccharomyces cerevisiae*

G_1 progression in budding yeast includes two waves of G_1 cyclin (Cln) transcription; *CLN3* transcription peaks at the end of M and early G_1 phase and *CLN1 & CLN2* mRNA level peak in late G_1 (McInerny, Partridge et al. 1997). Cln3/Cdc28 is an unstable activator of the Swi4/Swi6 transcription factor complex (Sidorova and Breeden 2003). Activation of these factors results in increased expression of a large group of G_1/S genes, including CLN1 and CLN2, which enable budding and the G_1-to-S transition (Mendenhall and Hodge 1998; Iyer, Horak et al. 2001). This process is called "Start" in yeast which corresponds to the irreversible commitment to a new cell cycle in budding yeast (Taberner, Quilis et al. 2009). Activation of Cln1/Cdc28 & Cln2/Cdc28 at the "Start" promotes S phase Cdk activities that are needed to begin S phase (de Bruin, McDonald et al. 2004).

As the yeast cell approaches M phase, another gene regulatory protein, the Mcm1-Fkh1/2-Ndd1 complex, stimulates the expression of about 35 G_2/M genes, encoding M cyclin Clb2 and Cdc20. In this way Mcm-Fkh helps stimulating the M/Cdk activity required for mitotic entry and Cdc20, which will eventually be needed for mitotic exit (Morgan 2007).

In late mitosis, the activation of two regulatory factors, Swi5 and Ace2, leads to expression of M/G_1 genes. Important target of Swi5 and Ace2 is the gene encoding Cdk inhibitor Sic1. Therefore, at the end of mitosis, Clb-Cdc28 is inactivated by

ubiquitin-dependent proteolysis (APC/C complex). After cell division, the system has returned to a stable G_1 state of low Cdk activity, where it is ready to begin the cycle again (Costanzo, Nishikawa et al. 2004) (Nasmyth 1996).

In early G_1, the activity of Cdk is suppressed by three mechanisms: the low level of cyclin gene expression, the presence of Sic1 and cyclin ubiquitination by APC/C^{Cdh1}. These inhibitory factors do not prevent the growth-dependent accumulation of the G_1 cyclin Cln3, however, and thus they do not block the gradual increase in Cln3-Cdk1 activity in G_1 (Morgan 2007). Figure 2 summarizes the phase-specific cyclin/Cdk complexes acting during the cell cycle.

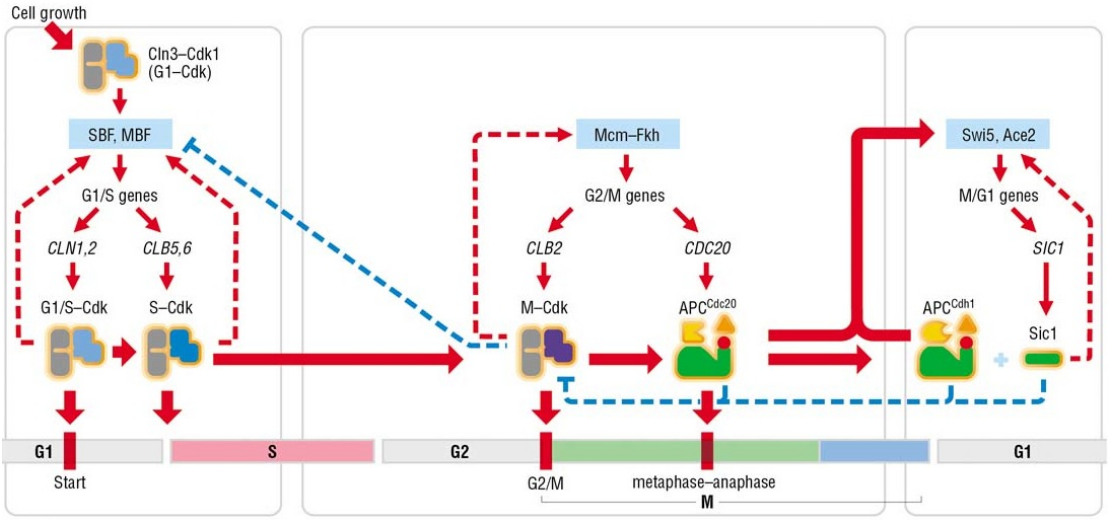


Figure 2: Overview of the cell cycle control system of budding yeast
Modified after (Morgan 2007)

4.1.3 Initiation of Replication

Eukaryotic cells regulate the replication of their genomes in a highly complex manner that ensures the accurate and fast duplication of genetic information. These regulatory mechanisms, including the sequence of events and the proteins involved, are highly conserved, although there are differences in the complexity of factors (Branzei and Foiani 2010). Replication initiates from multiple regions distributed along chromosomes. These so called replication origins share the following properties: Their DNA segments contain multiple short repeated sequences. These

sequences are recognized by multimer origin binding proteins that play a key role in assembling the replisome. Origins of replication contain AT rich sequences that facilitate unwinding of duplex DNA. *S. cerevisiae* have clear replication sequences called autonomously replicating sequences (ARS) (Zegerman and Diffley 2009). Initiation of DNA replication can be divided into three steps:

First, as cells exit mitosis, a large initiator protein complex, the prereplicative complex (pre-RC) assembles at origins. The central player in the assembly of pre-RC is a six-subunit complex called the origin recognition complex (ORC) (Alabert and Groth 2012).

Second, in G_1 phase, assembly of pre-replication complex begins when Cdc6 and Cdt1 associate with the bound ORC complex. This recruits another group of proteins called the MCM complex. Once the MCM2-7 rings are loaded onto DNA, the origin becomes licensed and ready to be activated (Mechali 2010).

Third, as cells enter S phase, activation of two S phase kinases, Cdc7 and Cdc28 triggers origin activation by promoting the formation of preinitiation complex (Remus and Diffley 2009). This, in turn, leads to the recruitment of large group of proteins including Cdc45-Sld3, GINS complex Dpb11-Sld2 and DNA polymerases to licensed origins (Koren, Soifer et al. 2010). During S phase, the MCM2-7 becomes activated in a reaction known as "origin firing" and starts unwinding of the DNA helix (Yekezare, Gomez-Gonzalez et al. 2013).

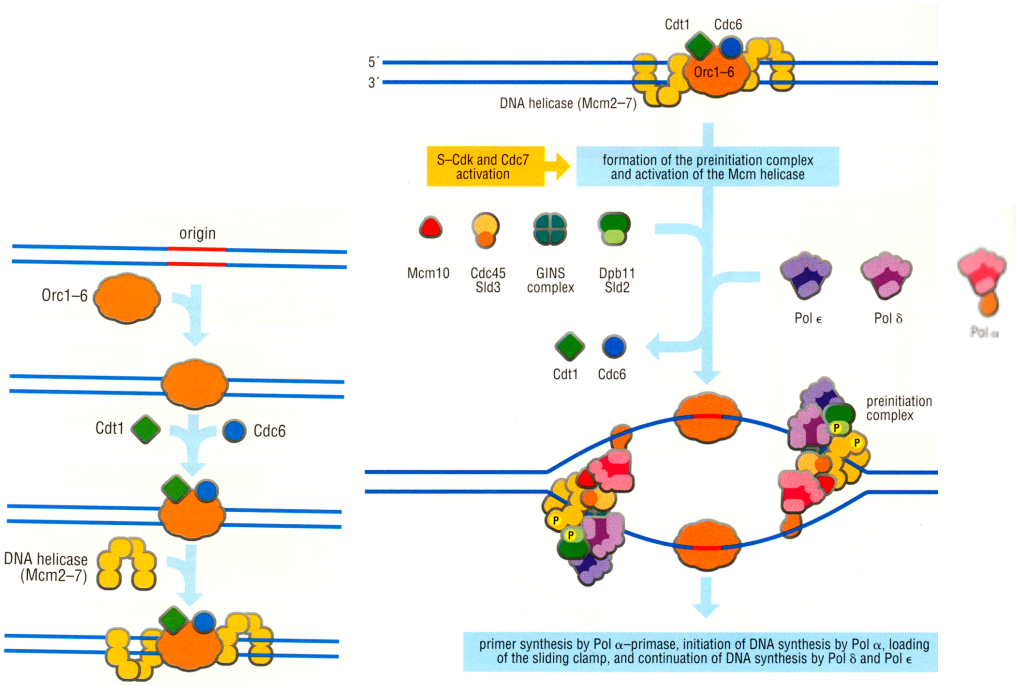


Figure 3: Initiation of Replication, Modified after (Morgan 2007)

4.1.4 DNA replication machinery

After initiation of DNA replication in the S phase, the MCM complex and Cdc45 form the active replicative helicase which moves away from replication origins as part of the DNA replication fork machinery. As the MCM complex unwinds the parental DNA, the GINS complex maintains protein-protein interactions within the replication complex – replisome in other words (Jones and Petermann 2012). The replisome also contains the clamp loader, RFC which helps to load the sliding clamp, PCNA. PCNA tethers the DNA polymerases to the chromosome, conferring processivity (Stukenberg, Studwell-Vaughan et al. 1991).

Since a DNA polymerase can only extend an existing DNA strand paired with the parental strand, a short fragment of DNA or RNA, called primer, must be created and paired with the template strand before a DNA polymerase can synthesize a new daughter strand. A primase tightly associated with polymerase α (Polα) synthesizes a primer that serves as starting point for the associated Polα. On the leading strand, binding of the ring clamp PCNA on the primer-template terminus displaces Polα. The association of polymerase ε (Polε) with PCNA stimulates its processivity, so that it can synthesize the remainder of the leading strand. DNA replication is believed to be continuous on the leading strand in contrast to the lagging strand where replication occurs in opposite direction of the replication forks under the control of Polδ. The lagging strand synthesis is carried out by the formation of Okazaki fragments which are processed and sealed later (Hubscher 2009).

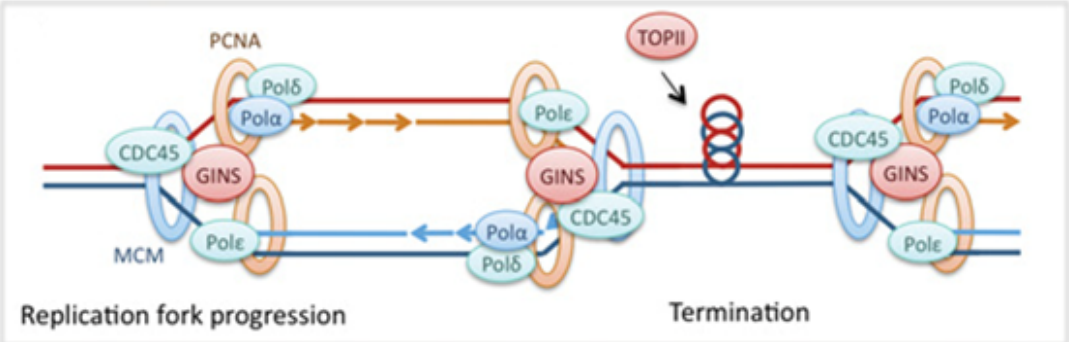


Figure 4: Overview of replication fork progression

Modified after (Jones and Petermann 2012)

Once in S phase, replisomes can encounter obstacles that hinder their progression and stability. These obstacles include DNA lesions, secondary DNA structures and large protein complexes bound to the DNA. In such cases, replication fork progression is blocked and the replisome is said to have "stalled" (Segurado and Diffley 2008). Replication fork stalling can also be caused by exogenous types of replication stress, such as inhibition of the replicative polymerases by aphidicolin or a reduction in the available dNTP pools by inhibition of ribonucleotide reductase (RNR) with hydroxyurea (HU) (Yekezare, Gomez-Gonzalez et al. 2013). It is critical to maintain an arrested replication fork in an appropriate configuration such that it can efficiently resume DNA synthesis once the obstacle is resolved. Failure to maintain an arrested fork in a stalled configuration results in fork collapse, a situation where resumption is impared (Hu, Sun et al. 2012). Molecular features of replication stress and the response pathways will be discussed further in the next chapters.

4.2 DNA Damage Response (DDR)

Growth and division of a single cell to yield two daughter cells requires the coordination of numerous events, in particular the faithful replication and partitioning of the cells genetic material to each daughter cell (Nyberg, Michelson et al. 2002). Since DNA is a large and complex entity, it is subject to a variety of chemical changes that are either spontaneous or catalyzed by the chemicals and radiation that bombard every cell (Khan, Moritsugu et al. 2000). DNA damage can take many forms, ranging from subtle changes in nucleotide base structure to breaks in both strands of the double helix, and it can occur at all phases of the cell cycle. All cells possess sensor proteins that can scan the genome, detect DNA damage and recruit specialized enzymes to repair it. If DNA is extensively damaged and not easily repaired, the damage sensors trigger a more extensive response called the DNA damage response (Nyberg, Michelson et al. 2002). Signaling pathways are activated that transmit the damage signal to a variety of effector proteins, some of which trigger increased production of DNA repair enzymes. Other effectors inhibit the cell cycle control system, thereby blocking cell cycle progression. This branch of the DNA

damage response is sometimes called the DNA damage checkpoint (Rouse and Jackson 2002). When DNA is damaged, this surveillance mechanism can delay G_1-to-S transition, decelerate S phase progression and let the cells pause before mitosis (Segurado and Diffley 2008; Jones and Petermann 2012).

4.2.1 DNA damage associated replication & repair

Under normal conditions the nucleotides in DNA are continually being modified by spontaneous hydrolysis and oxidation. Such reactions lead to several types of damages: depurination by hydrolysis of the bond connecting guanine or adenine bases to the nucleotide, deamination by hydrolytic attack on amino groups in cytosine, and alkylation. Environmental factors also contribute to DNA damage. UV radiation from sunlight causes covalent crosslinking of adjacent pyrimidine bases – producing thymine dimers. The detection and repair of altered nucleotide structure depends primarily on two major repair systems – base excision repair which finds relatively minor alterations in base structure and nucleotide excision repair which is responsible for the detection and repair of major modifications that alter the conformation of the double helix (Sancar, Lindsey-Boltz et al. 2004) (Hoeijmakers 2001).

Besides the environmental factors such as radiation and mutagenic chemicals, copying errors are occasionally introduced by polymerases during DNA replication. The fidelity of DNA replication is maintained by several distinct mechanisms, the first being the proofreading activity of the polymerases. Another mechanism that allows the removal of misincorporated bases that have not been detected and removed by the polymerase itself is mismatch repair. The mismatch repair machinery is a strand specific system for recognizing and repairing misincorporated bases that can arise during DNA replication. It consists of MutSα (Msh2/Msh6), MutSβ (Msh2/Msh3) and MutLα (Mlh1/Pms2). In *E.coli,* the hemimethylation pattern of newly synthesized daughter strands allows the mismatch repair system to excise the wrong incorporated base from the correct strand (Jiricny 2006). Gram-positive bacteria and eukaryotes do not use methylation in strand discrimination, and it was suggested that MMR might be directed to nascent DNA by strand discontinuities such as gaps between Okazaki fragments (Ghodgaonkar, Lazzaro et al. 2012). The different combinations of the MutS and MutL complexes confer additional repair features such as removing erroneous insertions or deletions, to this multifaceted

system (Jiricny 2006). Figure 5 summarizes the major responses to various DNA damages:

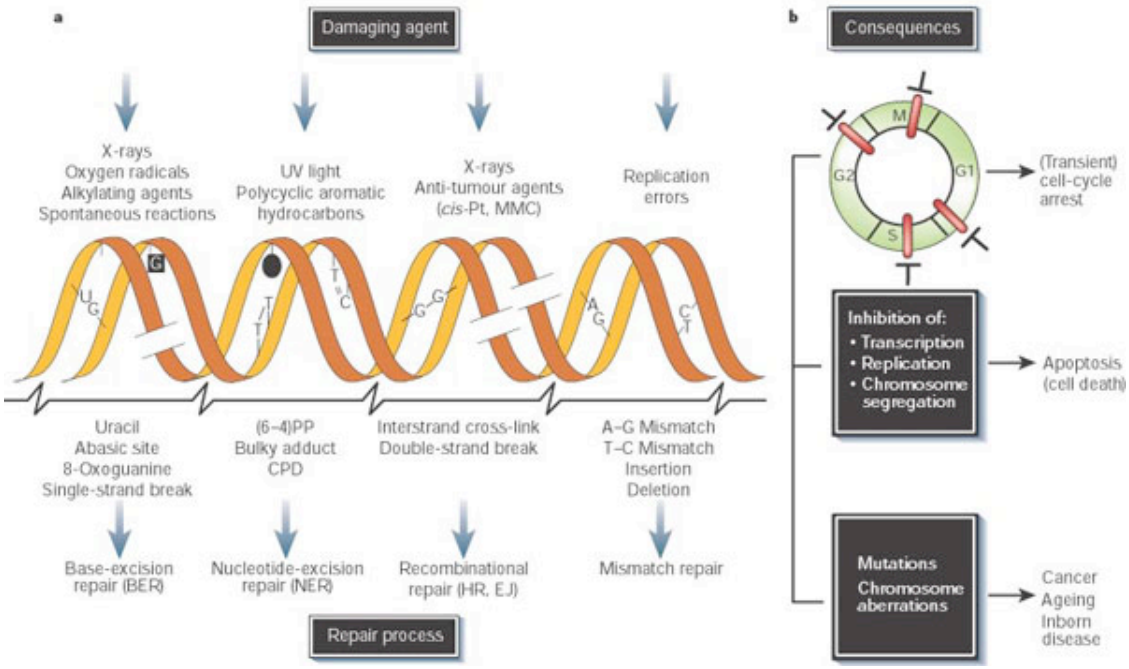


Figure 5: Summary of DNA damage and DNA repair pathways
Modified after (Hoeijmakers 2001)

The alterations that are discussed so far usually affect just one DNA strand at a given site, but both strands of the DNA double helix can also be broken as a result of exposure to DNA damaging agents, such as ionizing radiations (IR) and radiomimetic chemicals. These lesions, DNA double strand breaks (DSBs), can also occur during DNA replication, when DNA polymerase encounters a lesion in the template or secondary DNA structures (Longhese, Bonetti et al. 2010). Two different mechanisms address DSBs according to the cell cycle phase. During S and G2 phase of the cell cycle homologous recombination is the main pathway to repair these lesions and it is probably favoured because of the presence of the newly replicated sister chromatids, which provide a second copy of the sequence that can serve as template. Once a DSB occurs, the highly conserved Mre11, Rad50, Xrs2 (MRX) complex, composed of MRX subunits in budding yeast and MRN in mammals, is the first group of proteins

recruited to DNA ends. There, the MRX complex functions together with Sae2 protein in processing the DSB ends in 5`– 3` direction (Clerici, Mantiero et al. 2006) (Longhese, Bonetti et al. 2010). The 5` end can further be resected by the concerted action of helicase Sgs1 and two nucleases Exo1 and Dna2. This, in turn, generates long 3' single stranded tails from the previously created short ssDNA overhangs. These are the required substrates for binding of the Rad51 recombinase to initiate the homology search and strand invasion steps of recombination (Mimitou and Symington 2009) and for Rad52-mediated annealing (Mimitou and Symington 2008).

During G1 phase of the cell cycle, when a second copy is not available, the error prone non-homologous end joining (NHEJ) is the main pathway to address DSBs (Deriano and Roth 2013). In this approach the two broken ends are simply rejoined by DNA ligases; this process leads to loss of nucleotides at the repair site generally because the exposed ends of DSBs are resected and degraded by nucleases before being rejoined (Morgan 2007) (Deriano and Roth 2013). The molecular events during DSB repair are summarized in figure 6:

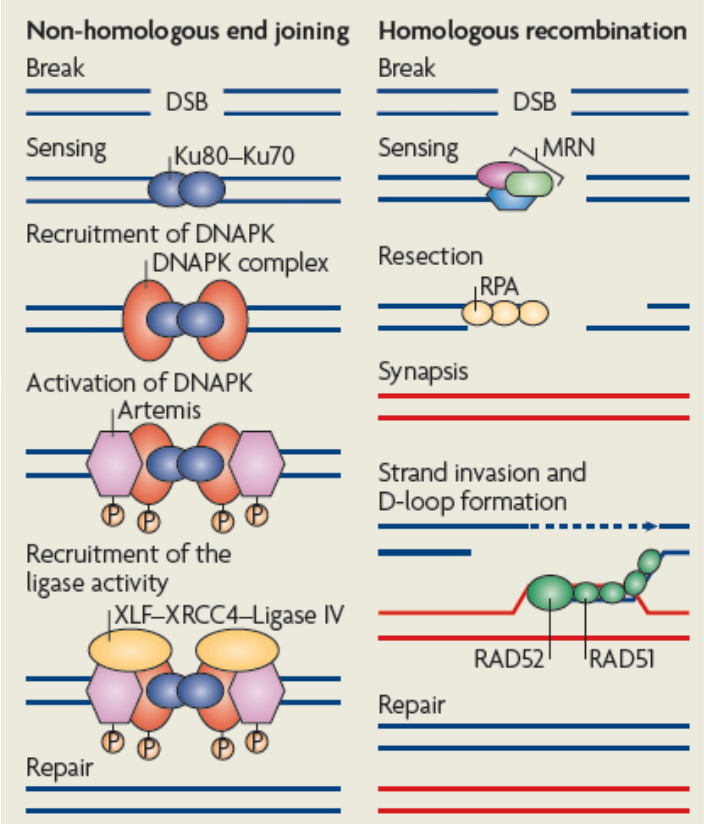


Figure 6: Major DSB repair pathways

Modified after (Misteli and Soutoglou 2009)

4.2.2 The checkpoint kinases

In all eukaryotes the DNA damage response is centered on the related protein kinases ATR and ATM and DNA-PK belonging to the subfamily of PIKKs (phosphatidylinositol 3`kinase-like kinases), whose sequence and function have been well conserved in evolution. Upon detection of the damage in *Saccharomyces cerevisiae*, Tel1 and Mec1 (yeast ATM and ATR, respectively) carry out the initial transduction of the DNA damage signal. Mec1 is part of a sensor mechanism that detects DNA damage in the form of single-stranded DNA and relays the checkpoint signal to a pair of transducing kinases, Rad53 and Chk1 (Harrison and Haber 2006). Among the two, Chk1 has a major role in metazoan checkpoints but a fairly minor role in budding yeast (Segurado and Diffley 2008).

Phosphorylation of downstream targets by the PIKKs facilitates physical protein interactions mediated by the phosphopeptide binding domains, FHA (forkhead-associated) and BRCT (Brca1 C terminus) that are found in numerous DNA damage-response proteins (Melo and Toczyski 2002).

Among such targets are adaptor protein Rad9 and effector kinases Chk1 and Rad53. Upon encountering DNA damage, Chk1 and Rad53 are phosphorylated by PIKKs. These downstream effector kinases undergo autophosphorylation, where FHA domains mediate self-oligomerization and interactions with mediator protein Rad9 to become fully active (Durocher, Smerdon et al. 2000; Durocher, Taylor et al. 2000). Rad53 then amplifies the signal and regulates the cell cycle machinery to effect checkpoint arrest prior to mitosis. The formation of these PIKK dependent protein complexes is the juncture at which the DNA damage or replication stress is amplified (Zhou and Elledge 2000).

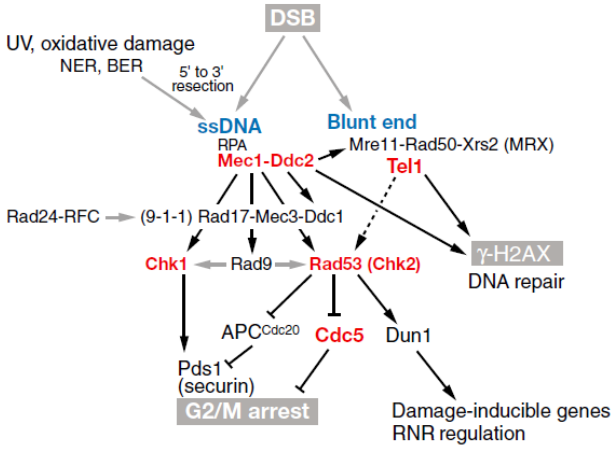


Figure 7: The DNA damage checkpoint in *S. cerevisiae*

Modified after (Harrison and Haber 2006)

	Budding yeast	Fission yeast	Human
PIKK	Mec1	Rad3	ATR
PIKK	Tel1	Tel1	ATM
Adaptor	Rad9	Crb2	53BP1, MDC1, BRCA1?
Rfc1 homolog	Rad24	Rad17	Rad17
9-1-1 clamp	Rad17	Rad9	Rad9
	Mec3	Hus1	Hus1
	Ddc1	Rad1	Rad1
MRX complex	Mre11	Mre11	Mre11
	Rad50	Rad50	Rad50
	Xrs2	Nbs1	Nbs1
BRCT domain adaptor?	Dpb11	Rad4/Cut5	TopBP1
Signaling kinase	Rad53	Cds1	Chk2
Signaling kinase	Chk1	Chk1	Chk1
Polo kinase	Cdc5	Plo1	Plk1
Securin	Pds1	Cut2	Securin
Separase	Esp1	Cut1	Separase
APC-targeting subunit	Cdc20	Slp1	$p55^{CDC}$/CDC20

Table 1: List of DNA damage checkpoint proteins

Modified after (Harrison and Haber 2006)

4.2.3 DNA replication checkpoint

Budding yeast cells are generally more sensitive to DNA damage in S phase than they are in G_1. The regulation of DNA replication by checkpoint controls is of key importance for the maintenance of genome stability and, potentially, in cancer therapy (Zhou, Anderson et al. 2003). Eukaryotic cells, therefore, respond to replicational interference through a complex network of signal transduction pathways known as the S phase checkpoint or the DNA replication checkpoint (Longhese, Bonetti et al. 2010). During S phase, the proteins that assemble at the replication fork serve not only as the machinery of DNA synthesis but also important sensors of many forms of DNA damage. When a traveling replication fork encounters damaged DNA its progress can be delayed or even blocked. The stalled replication fork then initiates DNA replication checkpoint that has at least three major outcomes:

i. Replication origins that have not yet been used are prevented from firing, thereby avoiding the initiation of further synthesis until the damage has been repaired.

ii. Progression through mitosis is blocked, ensuring that damaged chromosomes are not segregated.

iii. Replication forks are stabilized, allowing the safe resumption of DNA synthesis when damage has been repaired (Branzei and Foiani 2005) (Morgan 2007).

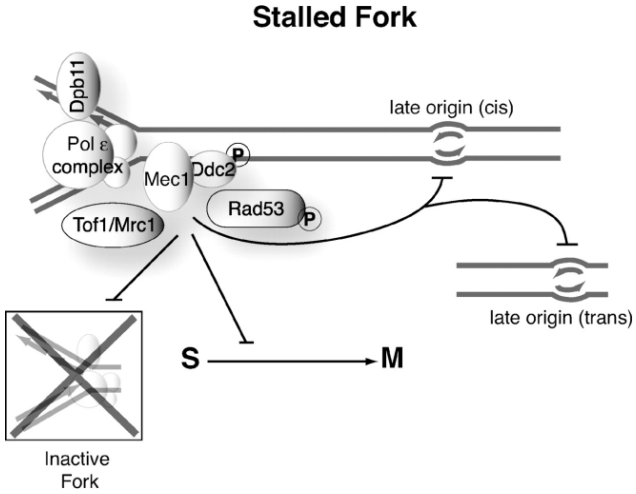


Figure: 8 Events occurring at stalled replication forks

Modified after (Nyberg, Michelson et al. 2002)

The first step in S phase checkpoint activation is stalling of replication forks owing either to the depletion of dNTPs or to an encounter with DNA adducts. Depletion of the nucleotide pool can be achieved by HU treatment, which causes stalling of the replication forks without inducing DSBs (Nyberg, Michelson et al. 2002). There are different types of independent molecular complexes that sense and signal different types of damage, of which the RPA-coated ssDNA is a central player (Branzei and Foiani 2008). Once formed ssDNA-RPA complex plays two crucial roles in recruiting Mec1-Ddc2 and the clamp loader Rad24 (Branzei and Foiani 2009).

The current model suggests that Mec1 does not recognize the primary lesion itself, but long stretches of ssDNA, which are generated either by functional uncoupling of replicative helicases and polymerases during fork stalling or nucleolytic processing of of DSBs (Paulsen and Cimprich 2007; Friedel, Pike et al. 2009).

However, an interesting concern could be the possible existence of checkpoint activating signals during an unperturbed cell cycle in the absence of external cues. DNA replication *per se*, for instance, generates a number of intermediates, that can mimic DNA structures arising as a consequence of damage. In this context, it is evident to point out that some checkpoint proteins are transiently phosphorylated during S phase and this modification requires a functional DNA damage checkpoint pathway (Paciotti, Lucchini et al. 1998; Foiani, Pellicioli et al. 2000).

Obvious checkpoint target candidates are the components of the replication machinery, which promotes the stabilization of the replisome with the fork:

i. Phosphorylation of Polymerase α seems to be important to stabilize the replisome (Lemoine, Degtyareva et al. 2005).
ii. Mec1-dependent phosphorylation of RPA has a role in replication stress response, although the exact function is still unknown (Zou and Elledge 2003).
iii. Mrc1 is associated with the replication fork under normal replication conditions, and, when cells are treated with HU, forms a pausing complex with Tof1 (Katou, Kanoh et al. 2003).
iv. The MCM-helicase complex has always been an attractive target candidate, since disassembly of it from stalled replication forks causes an irreversible HU arrest (Labib, Tercero et al. 2000).

Besides replisome components, increasing evidence indicates that the checkpoint targets chromatin remodelers and histone-regulating enzymes, factors that have an important impact on the maintenance of functional DNA replication forks (Segurado and Tercero 2009).

Recent studies put the nuclease Exo1 at the center of the checkpoint response at replication forks, as fork collapse in *rad53-null* mutants exposed to DNA damaging agents is dependent on Exo1 (Segurado and Diffley 2008). Furthermore, it has been shown that fork collapse in *rad53* cells blocked with HU also relies on Exo1 (Cotta-Ramusino, Fachinetti et al. 2005).

Rad53, together with Mec1, is required for the phosphorylation of Dun1, a protein kinase that controls several DNA damage inducible genes as well as genes encoding RNR subunits (Branzei and Foiani 2006). The checkpoint response during S phase is depicted schematically in figure 9.

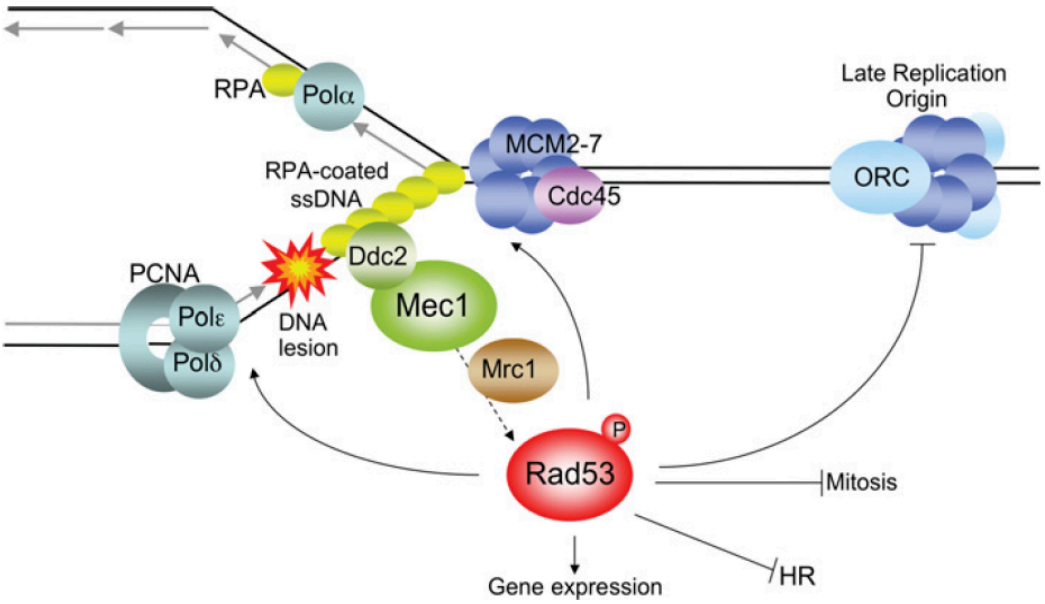


Figure 9: Schematic illustration of the S phase checkpoint response

Modified after (Segurado and Tercero 2009)

4.3 DNA Polymerases

DNA polymerases are DNA-dependent nucleotidyltransferases that replicate double stranded DNA in semi-conservative manner. These enzymes are required to duplicate the genetic material prior to cell division. Replication of chromosomes containing the anti-parallel strands of duplex DNA occurs through the copying of leading and lagging strand templates (Waisertreiger, Liston et al. 2012). DNA polymerases are also required for DNA repair, recombination, and translesion DNA synthesis to preserve genomic integrity (Kawasaki and Sugino 2001).

Since the discovery in 1957 of an enzyme that catalyzes the accurate replication of DNA, there has been a progressive accumulation of evidence for DNA polymerases. In human cells, there are at least 14 DNA polymerases that play a part in a wide variety of activities in the replication and maintenance of the genome (Shcherbakova, Bebenek et al. 2003) (Loeb and Monnat 2008).

4.3.1 Overview of Yeast DNA polymerases

In the yeast *S. cerevisiae,* a variety of biochemical and genetic approaches have been used to investigate the function of DNA polymerases. It is generally accepted that replicative DNA polymerases, responsible for chromosomal DNA replication, are essential for cell viability, but that DNA polymerases that are involved in other biological processes may be dispensable for cell growth (Kawasaki and Sugino 2001). Chromosomal DNA replication occurs by a trio of polymerases α, δ and ε with high fidelity (up to 10^{-11} per base replicated) due to three sequential fidelity control steps: base selection by DNA polymerases, exonucleolytic proofreading and DNA mismatch repair (Kunkel and Bebenek 2000; Waisertreiger, Liston et al. 2012). Their catalytic subunits possess the same general arrangement of essential motifs on the primary amino acid sequence (Tahirov, Makarova et al. 2009). In addition to the polymerase domain, Polδ and Polε also have relatively conserved sequences for the 3' → 5' exonuclease domains. Polα however lacks exonuclease activity because the sequence of catalytic motif in the Exo domain is altered and catalytic residues are missing (Waisertreiger, Liston et al. 2012).

Translesion DNA synthesis is an important process that allows the replication fork to bypass DNA lesions that are not removed prior to S phase. TLS polymerases synthesize DNA with high error rate and are responsible for introducing mutations into the genome during DNA damage bypass, so their replacement of the replicative polymerase must be tightly controlled (Daraba, Gali et al. 2014). During replication of damaged templates, internal gaps are likely generated by repriming downstream of the lesion on both leading and lagging strands (Heller and Marians 2006). These gaps can then be filled in by several different mechanisms. One pathway uses a combination of replicative and translesion synthesis polymerases to replicate across a lesion, a mechanism that can sometimes be error-prone (Branzei and Foiani 2009). Another gap filling mechanism, referred to as template switch, is essentially error-free and uses the undamaged information of the sister duplex in a homologous recombination (HR)-like manner (Branzei and Foiani 2007). Full understanding of the polymerase switch at DNA damage site is, however, still elusive. Table 2 summarizes the major DNA polymerases in budding yeast, together with their general features.

DNA polymerase	Gene	Essential?	Function	Homologue
α (I)	*POL1*	Yes	Chromosomal DNA replication	DNA polymerase α
	POL12	Yes		
	PRI1	Yes		
	PRI2	Yes		
ε (II)	*POL2*	Yes	Chromosomal DNA replication	DNA polymerase ε
	DPB2	Yes	and repair	
	DPB3	No		
	DPB4	No		
δ (III)	*POL3*	Yes	Chromosomal DNA replication	DNA polymerase δ
	POL31	Yes	and repair	
	POL32	No		
β (IV)	*POL4*	No	Double-strand break repair	DNA polymerase β
ϕ (V)	*POL5*	Yes	rRNA synthesis?	Pol5 in S. pombe
γ	*MIP1*	No	Mitochondrial DNA replication	DNA polymerase γ
ζ	*REV3*	No	Error-prone translesion DNA synthesis	DNA polymerase ζ
	REV7	No		
η	*RAD30*	No	Error-free translesion DNA synthesis	DNA polymerase η,κ,ι
Rev1	*REV1*	No	Deoxycytidyl transferase Translesion	Rev1
			DNA synthesis	

Table 2: DNA polymerases in *S. cerevisiae*
Modified after (Kawasaki and Sugino 2001)

4.3.2 Replicative DNA polymerases

Polα is a four-subunit complex that is responsible for the initiation of Okazaki fragments by concerted action of primase and polymerase α. Pol1 and Pri1 are the catalytic subunits while Pol12 and Pri2 both lack known enzymatic functions (Foiani, Lucchini et al. 1997; Garg and Burgers 2005). Two-hybrid assays have shown that the C-terminus of Pol1 is essential for the interaction with Pol12 (Biswas, Khopde et al. 2003).

Polδ in *S. cerevisiae* is a complex of three polypeptides consisting of Pol3, Pol31 and Pol32 (Garg and Burgers 2005). One distinguishing characteristic of Polδ is that its processivity and robust activity is contingent upon PCNA and accessory factors (Waisertreiger, Liston et al. 2012). Pol3 is the essential, catalytic subunit of the polymerase and it contains both the exonuclease and polymerase activity domains. The subunits of Polδ are held together by interactions with Pol31 (Burgers and Gerik 1998). The Pol32 subunit is not essential in *S. cerevisiae;* yet it is important for interaction with PCNA. Biochemical studies have shown that Polδ can interact with

PCNA through other sites positioned on the remaining subunits (Eissenberg, Ayyagari et al. 1997; Johansson, Garg et al. 2004). Pol32 has also been found to interact with the catalytic subunit of Polα - Pol1 (Johansson, Garg et al. 2004).

DNA Polε in *S. cerevisiae* is a four-subunit complex consisting of Pol2, Dpb2, Dpb3 and Dpb4 (Chilkova, Jonsson et al. 2003). *POL2* and *DPB2* are essential for cell viability while *DPB3* and *DPB4* are not. The subunits of Polε can be overexpressed and purified as two separate complexes, Pol2p-Dpb2p and Dpb3p-Dpb4p (Tsubota, Tajima et al. 2006).

Under normal circumstances, robust chromosomal replication in eukaryotes requires three DNA polymerases – Pol α, Polε and Polδ. DNA polymerases, however, cannot begin synthesis without primers. Therefore, during replication, primase produces short stretches of RNA primers. Primase is able to count the length of RNA primers by itself and synthesizes 8-12 nucleotide-long RNA primers and multiples of this unit (Waisertreiger, Liston et al. 2012). The problem of switching from synthesis of RNA to synthesis of DNA is solved by Polα that works in tight complex with primase (Muzi-Falconi, Liberi et al. 2003). Polα is not processive; yet extends these RNA primers by approximately 20-40 nt of DNA before Polα dissociates from the template (Garg and Burgers 2005). A switch then occurs to start the processive synthesis of numerous Okazaki fragments by Polδ or, rarely, by Polε, which continues with the bulk replication. These events in a progressing replication fork are summarized in figure 10.

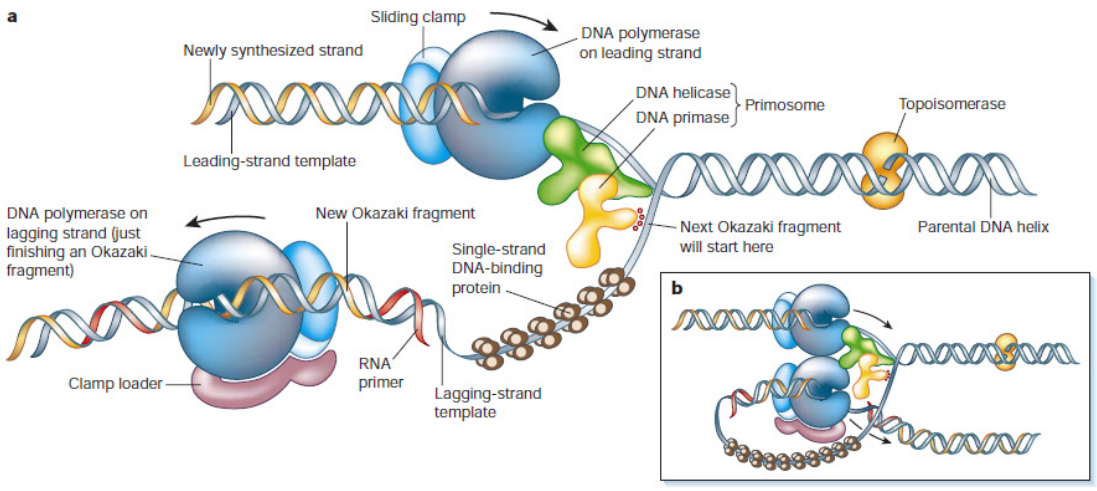


Figure 10: Schematic model of leading and lagging strand synthesis

Modified after (Alberts 2002)

Major types of proteins that act at a replication fork are illustrated simply in a. However, current view supports the close association of all these protein components moving in concert (shown in b).

The asymmetric nature of the DNA duplex poses topological problems for the replication of the anti-parallel strands by a fork moving in one direction. So the replication of the two strands is inherently different (Waisertreiger, Liston et al. 2012) and summarized in figure 11. DNA Polα–primase initiates synthesis of a short DNA fragment (blue arrow) that is primed by RNA (blue zigzag line) at the replication origin. The DNA fragment is then elongated by Pol δ/ε (leading strand, red arrow and lagging strand, green arrow). The lagging strand in *S. cerevisiae* is replicated in stretches of 150–200 nucleotides of Okazaki fragments. When the Okazaki fragment comes to an end—i.e. the polymerase meets a primer originating from a previously replicated fragment—2 to 3 nucleotides are displaced from the downstream primer, creating a 5'-flap containing the RNA. The flap is continuously removed by flap endonuclease (FEN1) leaving a nick in the DNA. The RNA primer must be removed before completion of the lagging strand. The nick is finally sealed by DNA ligase I to give a continuous DNA strand. The entire process is called Okazaki fragment maturation (Garg, Stith et al. 2004; Stewart, Campbell et al. 2006).

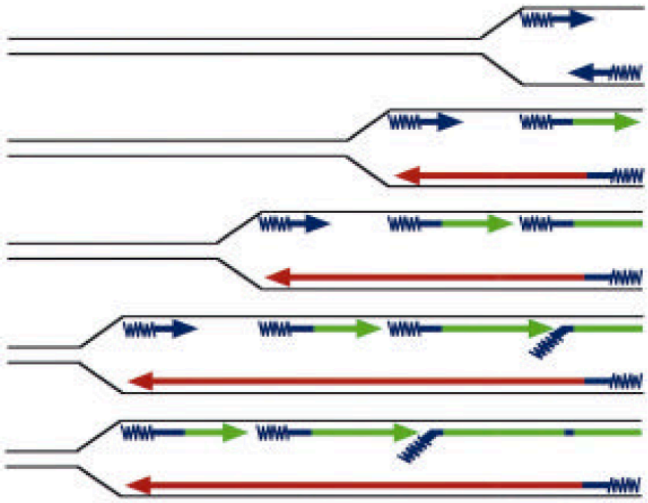


Figure 11: Model for chromosomal DNA replication

Modified after (Kawasaki and Sugino 2001)

4.3.3 DNA Polymerase ε

DNA polymerase ε (Polε) was identified 25 years ago as a DNA repair factor, but was soon recognized to play critical roles in replication, repair and cell cycle control (Henninger and Pursell 2014). All Polε enyzmes discovered to date consist of the same basic architecture. The core of the holenzyme is the large, catalytic subunit that can be divided into two subdomains: the N-terminal portion of the molecule is the catalytic domain and contains the polymerase and exonuclease active sites, while the C-terminal domain is catalytically inactive and appears to play a structural role in the enzyme (Hogg and Johansson 2012). The biochemical properties of Polε are quite similar to those of Polδ, although Polε is more processive than Polδ in the absence of cofactors (Kawasaki and Sugino 2001).

Pol2 is the catalytic subunit of Polε, and contains robust processive DNA polymerase and the proofreading exonuclease activities in the N-terminal domain (Bermudez, Farina et al. 2011). Despite this, it is only the C-terminal half of the subunit that is essential for cell survival. Cells completely lacking the N-terminal encoding part of *POL2* are viable but have impaired growth (Dua, Levy et al. 1998; Kesti, Flick et al. 1999). The very C-terminal end of the protein contains two putative zinc finger domains (Hogg and Johansson 2012), which are important for the response to DNA damage (Navas, Zhou et al. 1995; Dua, Levy et al. 1999) as well as for the interaction between Pol2 and Dpb2 (Dua, Edwards et al. 2000).

Like Pol2, Dpb2 subunit is also essential for cell viability. In budding yeast, Cdc28 phosphorylates Dpb2 during late G1 phase. This phosphorylation has been suggested to regulate its interaction with Pol2 and/or the activity of the Polε holoenzyme (Kesti, McDonald et al. 2004). Cells bearing mutant Dpb2 that weakens interaction with Pol2 confer a mutator phenotype, which is increased synergistically when either proofreading or mismatch repair are not operating (Jaszczur, Flis et al. 2008; Jaszczur, Rudzka et al. 2009). On the other hand, another study based on *in vitro* assays showed that Polε does not depend on Dpb2 for the synthesis of new DNA, proposing that the essential function of Dpb2 is separate from the enzymatic activity of Polε (Isoz, Persson et al. 2012).

The other two subunits Dpb3 and Dpb4 are not essential for cell viability in *S. cerevisiae.* The primary amino acid sequence of them suggests that the two small subunits contain histone fold motifs (Li, Pursell et al. 2000; Ohya, Maki et al. 2000)

which enables Dpb3 and Dpb4 form a complex that has affinity for dsDNA. Polε has the ability to bind dsDNA with high affinity, a property not normally associated with DNA polymerases (Tsubota, Maki et al. 2003). Subsequent work showed that the Dpb3/Dpb4 dimer acts in concert with the Pol2/Dpb2 heterodimer to bind dsDNA with an affinity much higher than the individual heterodimers (Tsubota, Tajima et al. 2006). Dpb4 also has another partner, Dls1, in a chromatin remodeling complex where it is involved in chromatin remodeling and epigenetic silencing of telomeres (Tsubota, Tajima et al. 2006), (Iida and Araki 2004).

Deletion of *DPB3* was previously shown to result in a modest mutator phenotype with slightly elevated spontaneous frameshift and base substitution rates *in vivo.* Dpb3 and Dpb4, however, offer no enhancement to the catalytic activity of Polε. Recent studies favor the conclusion that Dpb3 and Dpb4 do not directly influence replication fidelity *per se*, but rather contribute to normal replication fork progression by stabilizing the interaction of Polε with primer-template DNA and therefore affecting the processivity of the polymerase and exonuclease activities of Polε (Araki, Hamatake et al. 1991; Aksenova, Volkov et al. 2010), (Ohya, Maki et al. 2000).

The cryo-EM structure shows that the polymerase is built up of two structural domains: the head domain consisting of Pol2, and the tail domain consisting of Dpb2, Dpb3, and Dpb4. The tail domain has been shown to be important for the high processivity of the polymerase (Hogg and Johansson 2012), (Asturias, Cheung et al. 2006).

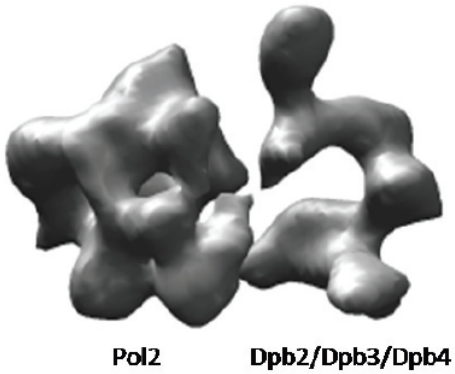


Figure 12: Cryo-EM Structure of Polε

Modified after (Asturias, Cheung et al. 2006)

Pol2 constitutes the head domain, while Dpb2/Dpb3/Dpb4 makes up the flexible tail domain.

Additional studies have been carried out to try to elucidate the separate roles of Polδ and Polε at the replication fork. By studying the effects in exonuclease-deficient yeast strains, it was concluded that the two polymerases proofread different strands (Shcherbakova and Pavlov 1996; Karthikeyan, Vonarx et al. 2000). Studies have shown that the two polymerases interact differently with PCNA (Eissenberg, Ayyagari et al. 1997), (Garg and Burgers 2005). Polδ interacts with PCNA through a C-terminal PIP box, whereas DNA synthesis by Polε is stimulated by PCNA to a lesser degree than Polδ. Deletion of the PIP motif, moreover, was not found to affect cell viability (Garg and Burgers 2005). Since the dependence on PCNA for processivity has been proposed to be a factor characteristic of a lagging-strand polymerase (Garg and Burgers 2005), it was suggested that Polδ is involved in the synthesis of lagging strand. Polδ, in contrast to Polε, functionally interacts with Fen1 and DNA ligase I during the processing of primers in the Okazaki fragments (Jin, Obert et al. 2001), (Garg, Stith et al. 2004), which further supports the hypothesis that Polε is not a lagging strand polymerase.

However, the current model does not exclude the possibility of Polδ on the leading strand. In fact, the observation that cells completely lacking the N-terminal encoding part of *POL2* are viable, suggests that another polymerase, presumably Polδ, can compensate for the loss of Polε catalytic activity (Dua, Levy et al. 1999).

Since Polε was first identified as a polymerase essential for DNA replication, it has been suggested to have several roles during the course of the cell cycle. ChIP assays have shown that Polε is loaded at the origin of replication, both in early and late S phase, indicating that it plays some parts during the initiation of DNA replication (Aparicio, Stout et al. 1999). Polε participates in a pre-loading complex consisting of Polε, GINS, Dpb11, and Sld2 (Muramatsu, Hirai et al. 2010).

Apart from the replicative role, Pol ε is also an important factor in DNA repair mechanisms. It was shown to be involved in homologous recombination, mismatch repair, and nucleotide excision repair (Shcherbakova, Bebenek et al. 2003).

As discussed in previous chapters, uncoupling of the helicase from the replicative polymerases is suggested to generate large amounts of ssDNA that recruits RPA and triggers signaling pathways (Navadgi-Patil and Burgers 2009). During chromosomal replication, the lagging strand has always a certain amount of ssDNA due to the synthesis of the Okazaki fragments. In contrast, it is less likely to find significant amounts of ssDNA on the leading strand during normal replication. Thus, the leading

strand polymerase is ideally positioned to participate in the sensory mechanism for the generation of checkpoint signals. Indeed, the *pol2-12* allele in budding yeast with a premature stop codon at the C-terminus of Pol2, fails to activate Dun1 kinase, transcription of *RNR3* in response to DNA damaging agents and enter into mitosis before completion of DNA replication (Navas, Zhou et al. 1995). Another model is proposed in which stalling of leading strand synthesis by Polε signals the Dpb11/Sld2-Mec1-Rad53 signaling cascade. This activity appears to be dependent on Dpb4 and suggests that leading and lagging strands sense DNA damage and signal this via different pathways (Puddu, Piergiovanni et al. 2011). This is an interesting model since Polε is inhibited by ssDNA, while Polδ is less sensitive to the presence of ssDNA (Chilkova, Stenlund et al. 2007).

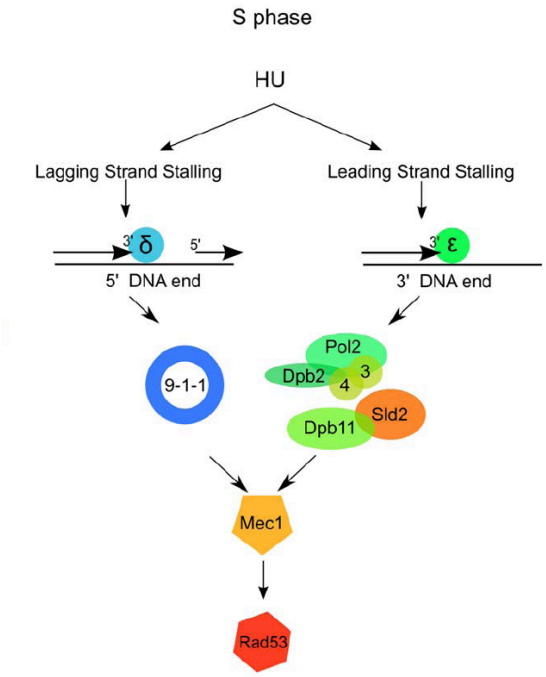


Figure 13: A model for 9-1-1 and Polε mediated S phase checkpoint activation

Modified after (Puddu, Piergiovanni et al. 2011)

In S phase Dpb11 and the 9-1-1 complex signal replication stress to Mec1 independently from each other, likely because the detection of replication stress occurs independently on the leading and lagging strands. 9-1-1 complex could signal replication stress on the lagging strand, where the 5' ends necessary for its loading are generated as the result of discontinuous replication. Dpb11, instead, could signal replication stress on the leading strand together with the interacting Polε.

4.4 14-3-3 Proteins

14-3-3 proteins form a family of highly conserved, acidic, dimeric proteins with a subunit mass of 28-33 kDa (van Heusden 2009). These proteins were originally described as abundant brain specific proteins. Later it was found that they are present in all eukaryotic cells and tissues. Many organisms contain multiple isoforms: Seven 14-3-3 isoforms exist in mammalian cells - β, γ, ε, σ, ζ, τ, η. On the other hand, the model plant *Arabidopsis thaliana* contains at least 13 isoforms (Morrison 2009; van Heusden 2009).

The budding yeast *S. cerevisiae* has two genes encoding 14-3-3 proteins – namely *BMH1* (Brain Modulosignaling Homologue 1) and *BMH2* (van Heusden and Steensma 2006). Disruption of one of the genes has little effect on cell viability, whereas the simultaneous disruption of both genes is lethal in most of the yeast strains.

4.4.1 Structure

The 14-3-3 proteins form stable homo- and heterodimers that can interact with a wide range of cellular proteins. Each monomer consists of a bundle of nine anti-parallel α-helices. The 14-3-3 protein dimer possesses a characteristic cup-like shape with a central channel containing two binding grooves. The inner walls of the central channel and the dimer interface are formed by conserved residues, whereas the less conserved residues are located on the outer convex surfaces (Obsilova, Kopecka et al. 2014).

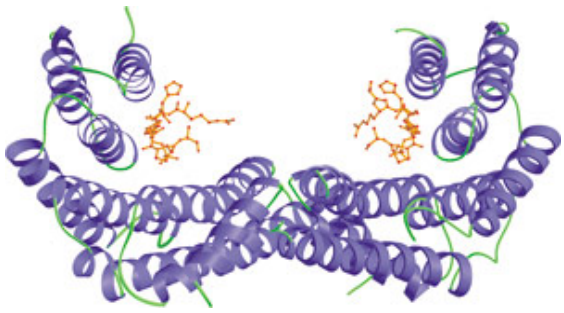


Figure 14: The structure of a 14-3-3 dimer bound to the indicated (red) phosphoserine peptides

Modified after (Dougherty and Morrison 2004)

The most flexible region of the 14-3-3 protein molecule is the C-terminal segment which also exhibits highest sequence variability among different 14-3-3 isoforms. This region, due to its flexibility, has been shown to play an important role in the regulation of binding properties of the 14-3-3 protein isoforms (Silhan, Obsilova et al. 2004; Obsilova, Kopecka et al. 2014). Like the C-terminal region, the amino terminal region of 14-3-3 proteins is not highly conserved. This region is responsible for the dimerization of 14-3-3 proteins (Bridges and Moorhead 2005). The extensive interactions between α-helices make the structure of the dimer highly rigid which suggests that the 14-3-3 protein can behave as a rigid platform on which the bound target protein can be reshaped (Yaffe 2002) (Obsilova, Kopecka et al. 2014).

4.4.2 Function

The highly conserved eukaryotic 14-3-3 proteins establish phosphorylation-dependent interactions with more than 200 different cellular proteins. These proteins specifically recognize phosphoserine/phosphothreonine-containing motifs. Three types of consensus 14-3-3 binding motifs were identified: R[S/Φ][+]pSXP (mode I), RX[S/Φ][+]pSXP (mode II) and pS-X_{1-2}-COOH (mode III) where pS is phosphoserine (the phosphorylated residue can also be threonine), Φ is an aromatic residue, + is a basic residue and X is any type of residue (Ganguly, Weller et al. 2005) (Obsilova, Kopecka et al. 2014). Many target proteins, however, do not contain sequences that conform precisely to these motifs. This may relate to the dimeric structure of 14-3-3 proteins which argues that they usually recognize their target proteins in a specific manner via a discrete phosphoserine or phosphotreonine motif (Bridges and Moorhead 2005).

In addition, “imperfect” sites may be sufficient to bind to the 14-3-3 dimer and phospho-independent interactions, which do not require the above mentioned consensus sequence, have additionally been reported (Bridges and Moorhead 2005). To date, nonphosphorylated targets constitute a very small population of the total number of 14-3-3 interactors.

14-3-3 binding can have various consequences for the binding partner. The mechanism of 14-3-3 protein function can be generally classified into three modes of action: (A) 14-3-3 proteins can induce direct conformational changes to their target proteins; (B) 14-3-3 binding can occlude a specific region on the target and likewise

mask for instance an active site and (C) binding of 14-3-3 proteins can lead to the colocalization of two proteins, where the 14-3-3 dimer acts as a docking platform (Bridges and Moorhead 2005).

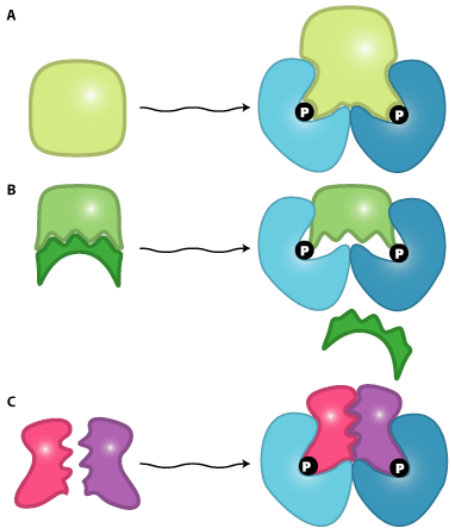


Figure 15: Structural effects of 14-3-3 binding

Modified after (Bridges and Moorhead 2005)

4.4.3 Roles

Given that phosphorylation is a key regulatory mechanism in cell biology and 14-3-3 interactions are primarily phosphorylation-dependent, the 14-3-3 proteins are involved in many important cellular processes such as metabolism, apoptosis, transcription, sporulation, cytoskeletal organization, protein trafficking, vesicle targeting, signal transduction, glucose metabolism, bud morphogenesis and cell-cycle (Morrison 2009).

In addition, 14-3-3 proteins can also bind cruciform DNA, a structure that was shown to form at yeast origins of replication (Yahyaoui, Callejo et al. 2007). 14-3-3 proteins are involved in DNA replication of eukaryotes through binding to the cruciform DNA that forms transiently at the replication origins at the onset of S phase. 14-3-3 proteins, through its association with replication initiation proteins such as Mcm2 and Orc2 (Yahyaoui and Zannis-Hadjopoulos 2009), might partake in the regulation of the initiation of DNA replication.

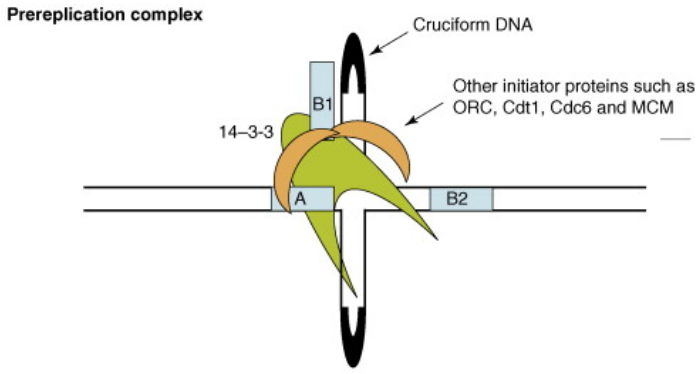


Figure 16: 14-3-3 Proteins can bind cruciform DNA

Modified after (Zannis-Hadjopoulos, Yahyaoui et al. 2008)

Upon DNA damage and DNA replication stress, 14-3-3 proteins are required for cell cycle restart, suppression of genomic instability and viability (Lottersberger, Rubert et al. 2003). Moreover, 14-3-3 proteins genetically and physically interact with the checkpoint protein Rad53 and genetically with the checkpoint kinase Dun1. The physical interaction with Rad53 only occurs in the presence of the DNA damaging agent MMS (methyl methanesulfonate). 14-3-3 proteins directly facilitate Rad53 function *in vivo* by stabilizing an active form of the kinase (Usui and Petrini 2007). These interactions may be an explanation for the previously observed checkpoint defects in 14-3-3 mutant yeast cells (Lottersberger, Rubert et al. 2003). The yeast 14-3-3 proteins were also shown to interact with the acetyltransferase and deacetylase Esa1 and Rpd3 upon replication perturbations (Lottersberger, Panza et al. 2007). Taken together, these data point to an important role of 14-3-3 during replication stress, although their exact mechanism of action in this context of remains unknown.

4.5 Aim of the study

Based on previous work on *rad53*-defective cells, stalled replication bubbles have been shown to accumulate long ssDNA regions. These lesions most likely arise by resection of the nascent chains by the Exo1 nuclease that is recruited to stalled replication forks where it counteracts fork reversal upon HU treatment (Cotta-Ramusino, Fachinetti et al. 2005) (Sogo, Lopes et al. 2002).

Recently published data from our laboratory provided evidence that 14-3-3 proteins are central regulators of events at sites of stalled replication forks and are especially important for the regulation of the elongation kinetics. We obtained evidence that 14-3-3-deficient yeast cells cannot efficiently restart replication forks upon recovery from HU. The data also indicated that in these cells ssDNA gaps accumulated behind the fork in an Exo1-dependent manner (Engels, Giannattasio et al. 2011). However, while deletion of the *EXO1* gene rescued the accumulation of ssDNA gaps, it was unable to rescue HU sensitivity and the slow fork progression/restart phenotype of 14-3-3-deficient cells. The fact that defective fork progression in 14-3-3-deficient cells is independent of Exo1 (Engels, Giannattasio et al. 2011), indicates that other proteins may play a role in the suppression of replisome restart. In analogy to Exo1, such factors may also be controlled by 14-3-3 proteins in conditions of replication stress.

The aim of this thesis is to assess the molecular mechanism by which 14-3-3 proteins control DNA replication. Due to the fact that 14-3-3 proteins have already established roles in human diseases (van Heusden and Steensma 2006), this study will provide more evidence for our knowledge on pathways controlled by 14-3-3 proteins. Considering that elucidating a particular mechanism in a model organism facilitates the identification of similar pathways in human cells (Paul, Templeton et al. 2014), this study will help expanding our knowledge on pathways that control processive DNA synthesis and maintain genomic stability. This, in turn, will offer novel targets for cancer therapy.

5. MATERIALS AND METHODS

5.1 *Saccharomyces cerevisiae* strains

Yeast strains were cultured at 28 ^{0}C in either YPD medium (1% bacto yeast extract, 2% bacto peptone, 2% glucose) or selective medium; SD-Ura containing 0.67% yeast nitrogen base, 2% glucose and amino acid supplements lacking uracil. YPD/Selection plates are supplemented with 2% agar. Yeast strains used in this study are all isogenic to *W303-1A* or *CML128.* They are listed below in table 3:

Table 3: Strains used in this study

Strain Name	Background	Genotype	Source
W303-1A		*MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 [phi+]*	R.Rothstein
YKE2	*W303-1A*	*MATa bmh2Δ::NAT1 bmh1Δ::HIS3::bmh1-280::LEU2*	M.P. Longhese
YKE15	*W303-1A*	*MATa BMH1-HA::URA3::bmh1 EXO1-Myc::KANMX4::exo1*	K. Engels
YKE19	*W303-1A*	*MATa dpb3Δ::KANMX4*	This study
YKE21	*W303-1A*	*MATa bmh2Δ::NAT1 bmh1Δ::HIS3::bmh1-280::LEU2 dpb3Δ::KANMX4*	This study
YKE22	*W303-1A*	*MATa BMH1-HA::URA3::bmh1 CHL1-Myc::KANMX4::chl1*	This study
YKE23	*W303-1A*	*MATa BMH1-HA::URA3::bmh1 DPB3-Myc::KANMX4::dpb3*	This study
YKE24	*W303-1A*	*MATa BMH1-HA::URA3::bmh1 RAD5-Myc::KANMX4::rad5*	This study
YKE39	*W303-1A*	*MATa BMH1-HA::URA3::bmh1*	M.P. Longhese
YKE78	*W303-1A*	*MATa RAD5+ rev7Δ::HIS3 rev3Δ::TRP1 rev1Δ::KANMX6 rad30Δ::KANMX6*	M. Giannattaso
YMA107	*W303-1A*	*MATa dpb4Δ::KANMX4*	This study
YMA108	*W303-1A*	*MATa bmh2Δ::NAT1 bmh1Δ::HIS3::bmh1-280::LEU2 dpb4Δ::KANMX4*	This study
YMA109	*W303-1A*	*MATa stm1Δ::KANMX4*	This study
YMA110	*W303-1A*	*MATa bmh2Δ::NAT1 bmh1Δ::HIS3::bmh1-280::LEU2 stm1Δ::KANMX4*	This study
YMA113	*W303-1A*	*MATa BMH1-STREP::KANMX4::bmh1*	This study
YMA118	*W303-1A*	*MATa BMH1-HA::URA3::bmh1 DPB3-STREP::KANMX4::dpb3*	This study
YMA120	*W303-1A*	*MATa bmh2Δ::NAT1 bmh1Δ::HIS3::bmh1-280::LEU2 sit4Δ::KANMX4*	This study
CML128		*MATa leu2-3,112 trp1 can1 ura3-52 his4*	C. Gallego
YMA122	*CML128*	*MATa, sit4Δ::KANMX4*	J. Arino
YMA134	*W303-1A*	*MATa RAD5+ rev7Δ::HIS3 rev3Δ::TRP1 rev1Δ::KANMX6 rad30Δ::KANMX6 dpb3Δ::URA3*	This study
YMA137	*W303-1A*	*MATa BMH1-HA::URA3::bmh1 SIT4-MYC::KANMX4::sit4*	This study

5.2 List of oligonucleotides and vectors

Table 4: Vectors and oligonucleotides used in this study

Name	Specification	Sequence	
MA1	PPH3 Forward	AACGCCGGATCCATGATGGACTTAGATAAGATTA TAGC	
MA2	PPH3 Reverse	AAACCTTGCGGCCGCTTATAAGAAATAGTCCATTT GAG	
MA3	Glc7 Forward	AACGTCGGATCCATGGACTCACAACCAGTTGACG	
MA4	Glc7 Reverse	AAACATTGCGGCCGCTTATTTTTCTTTCTACCCC CAGC	
MA12	Kanmx Fwd. Ctrl.	GGTCTAGAGATCTGTTTAGCTTG	
MA13	Forward BMH1 tag	CAGCAACAGCAGCCACCTGCTGCCGCCGAAGGTG AAGCACCAAAGCGTACGCTGCAGGTCGAC	
MA14	Reverse BMH1 tag	TTTCTTTTTTTTAGTAATTTCTCTTTAGATTTATCA GAATACTTAATCGATGAATTCGAGCTCG	
MA15	Forward STM1 deletion	GGTGAAGTAGAAATAAACCAAGAAAGCATACAC ATTTTATTCTCACAGCTGAAGCTTCGTACGC	
MA16	Reverse STM1 deletion	CACTGTTATTGGATTCTTTCAGTTGGAATTATTCA TATATAAGGCGCATAGGCCACTAGTGGATCTG	
MA25	Forward SIT4 tag	GAATCCACGGCAAACCATAATAATCAAAGAGCCG GCTATTTCTTACGTACGCTGCAGGTCGAC	
MA26	Reverse SIT4 tag	AATTATTTTTATTCGTCGAGTTAGGGAGGGCATGC CGTCGTGTTAATCGATGAATTCGAGCTCG	
MA27	Forward SIT4 deletion	GAAATACTATTGAAGCTCAAAAACATCCATAATA AAAGGAACAATAACACAGCTGAAGCTTCGTACGC	
MA28	Reverse SIT4 deletion	GAAAATTATTTTTATTCGTCGAGTTAGGGAGGGC ATGCCGTCGTGGCATAGGCCACTAGTGGATCTG	
MA31	Forward ctrl tagging	CGTACGCTGCAGGTCGAC	
KE27	Forward CHL1 tag	TCTTCAACACGGAAGTTTTTTTCAATGCGCAGCCT GAATTCACGCCGTACGCTGCAGGTCGAC	
KE28	Reverse CHL1 tag	ATATATAGTAGTAATCACAGTATACACGTAAACG TATTCCTTTTAATCGATGAATTCGAGCTCG	
KE31	Forward DPB3 tag	GACTCTTCTGATATCGAAGTTGACCATACGAAAA GCACCGATCCTCGTACGCTGCAGGTCGAC	
KE32	Reverse DPB3 tag	ATTGCATCGAATAGTAATTACATAGCAATAATAG CAACAACACTAATCGATGAATTCGAGCTCG	
KE35	Forward RAD5 tag	GAGAGAAGAAAAAGGAGAATTGAAGAAATCCAG ATGCTGTTTGAACGTACGCTGCAGGTCGAC	
KE36	Reverse RAD5 tag	ATAATAATAAATAAAGTCTTTATATATGAGTATGT GGTATGACTAATCGATGAATTCGAGCTCG	
KE59	Kanmx Rev. Ctrl	GCGAGACGAAATACGCGATCG	
Vectors			
Name	**Source**	**Description**	**Marker**
pYM18	Euroscarf	Tagging casette (9xmyc)	KanMX
S2	This study	PstI - Strep tag (1x) - NcoI in pYM18	KanMX
S3	This study	NcoI - ClonNat - EcoRV in S2	ClonNat
pUG6	Euroscarf	Deletion casette	KanMX
pUG72	Euroscarf	Deletion casette	URA3
pCM190	ATCC	Tet-inducible expression vector	URA3
pMA1	This study	BamHI - PPH3 ORF - NotI in pCM190	URA3
pMA2	This study	BamHI - GLC7 ORF - NotI in pCM190	URA3

5.3 Reagents

The antibodies used in this study were: mouse monoclonal anti-HA (12CA5, Sigma); mouse monoclonal anti-Myc (9E10, Santa Cruz Biotechnology) and goat polyclonal anti-Rad53 (yC-19, Santa Cruz Biotechnology).

The chemicals and peptides used in this study were: Hydroxyurea (Bio Basic), α1-Mating Factor (Primm, Milan, Italy), Methyl methanesulfonate (Sigma), Doxorubicin (LC Labs), GelRed (Biotium).

5.4 Methods

DNA gel electrophoresis

For DNA gel electrophoresis, 1% agarose gel was prepared in 1x TAE buffer (40 mM Tris-Acetate, 2 mM EDTA) containing ethidium bromide (0.5μg/ml final concentration). For electrophoretic analysis of gDNA, 0.8% agarose gel in 1x TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH: 8.0) containing 1x GelRed™ (Biotium) was preferred. As size marker, QuickLoad 1 kb and 100 bp DNA ladder (NEB) were used. The gels were run at 100-125V.

Molecular cloning

For cloning of Pph3, primer pair MA1/2 and for cloning of Glc7, primer pair MA3/4 were used in order to amplify the insert. Fragments were purified according to the PCR purification kit protocol (supplied protocol of NucleoSpin Gel/PCR cleanup kit – Macherey Nagel) and digested with BamHI/NotI (NEB) in 30 μl reaction mixtures. Ligation reactions were carried out in 20 μl of mixtures containing digested vector pCM190 and 1 μl of DNA ligase overnight at 16 ^{o}C. Ligation mixtures were then transformed into electrocompetent *E.coli DH10β*.

Yeast transformation

Saccharomyces cerevisiae cells in logarithmic phase (always OD_{600}<0.6) were transformed by lithium acetate method (Gietz and Schiestl 2007). Tagged/Deletion

strains were obtained by one-step replacement of the amplified casette. Accuracy of transformations has been verified by PCR and Western blot.

Genomic DNA extraction

Genomic DNA for cloning, and strain verification purposes as well as for generation of tools was performed as described earlier (Dichtl, Aasland et al. 2004). The DNA pellet was resuspended in 30-50 µl TE buffer and stored at 4 ^{o}C.

Cell synchronization, Flow cytometry

For block-and-release experiments, exponentially growing cells grown at 28°C were synchronized in G1 with 3 µg/ml α-factor. Cells were then released by adding 20 mg/ml Pronase E (Sigma).

The DNA content of individual cells was measured using CyAn ADP cell sorter. Cells fixed in 70% ethanol were resuspended in 50 mM Tris buffer (pH 7.5) and treated with 1 mg/ml RNase over night at 37°C. Cells were prepared for flow cytometry by staining them with propidium iodide (Molecular Probes).

HU, MMS, Doxorubicin sensitivity assays

Spot dilution assays were performed by plating 6 µl of serial 10-fold dilutions of a liquid culture containing 200 – 2x10^{6} cells/ml. Cells were spotted on YPD plates containing different HU/MMS/Doxorubicin concentrations and grown for 2-4 days.

2D gel electrophoresis

DNA extraction with the CTAB method and neutral-neutral two-dimensional gel electrophoresis were performed as described (Lopes, Cotta-Ramusino et al. 2003).

Protein extraction, western blotting, immunoprecipitation and pull-down assays

Western blot analysis of yeast proteins was carried out upon TCA extraction (Muzi Falconi, Piseri et al. 1993). Proteins were resolved on 10% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride (PVDF) (GE-Healthcare) and the membrane was probed with appropriate antibodies. Immune complexes were revealed using the enhanced chemiluminescence system (GE-Healthcare).

To visualize phospho-Dpb3, an optimized Phos-tag system (5 mM Phos-tag reagent) was employed according to (Kinoshita, Kinoshita-Kikuta et al. 2008). Proteins were transferred to nitrocellulose (porablot NCP, 0.45 µm pore size, Machery-Nagel) overnight at room temperature applying constant amperage (200 mA).

For immunoprecipitation, yeast proteins were extracted using ice-cold lysis buffer (25 mM Tris-HCl pH 7.4, 15 mM NaCl, 15 mM EGTA, 1mM NaF, 1mM Na orthovanadate, 4 mM p-Nitro-Phenyl-Phosphate (pNPP), 0.1% Triton X-100, 1mM PMSF, complete protease inhibitors cocktail (Roche)). Protein concentration was determined using the Bio-Rad Protein Assay Reagent (Bio-Rad). Dpb3-Myc was immunoprecipitated from 10 mg total cell extracts using the monoclonal antibody 9E10 to the Myc-tag. The antibody was captured for 2h at 4°C using protein A-agarose beads. Beads were washed in 4 x 1ml ice-cold lysis buffer and heated for 10 min at 95°C in 2x Laemmli sample buffer. Proteins were resolved on 7.5% or 10% SDS-polyacrylamide gels and detected as described above using anti-HA or anti-Myc monoclonal antibodies.

Dpb3-Strep and Bmh1-Strep were pulled down from 5 mg total cell extracts using *Strep*-Tactin® Sepharose beads (IBA Life Sciences).

Gap labeling assay

A modified version of previously described protocol was used for radioactive gap labeling (Fukui, Yamauchi et al. 2004). Purified genomic DNA was incubated with 4 units of T4 DNA polymerase (Fermentas) and [α-^{32}P] dATP in a buffer containing 67 mM Tris-HCl, pH 8.8, 6.6 mM $MgCl_2$, 16.8 mM ammonium sulphate, 1 mM dithiothreitol (DTT), 0.1 mg/ml BSA and 200 µM each of dNTP for 30 min at 37 °C. Labeled products were analyzed by alkaline and native agarose gel electrophoresis.

Mass spectrometric analysis

Bmh1-Strep was pulled down from 5 mg total cell extracts using *Strep*-Tactin® Sepharose beads. After the last wash of the beads, 100 µl of D-Desthiobiotin was added to the tubes. Upon incubation at 4 ^{0}C for 1h with vigorous shaking, proteins bound to beads were eluted and supernatants were recovered. 10% of the supernatants were run on SDS polyacrylamide gel and silver-stained.

The rest of the eluates were subjected to tri-chloroacetic acid (TCA) precipitation by addition of the acid to a final concentration of 20%. Samples were vortexed, incubated on ice for 2h, centrifuged and protein pellets were washed in 1 ml of 10% ice-cold TCA. Pellets were then washed with 1 ml of ice-cold HPLC-grade acetone, centrifuged and air dried completely. Protein pellets were stored at -80 ^{0}C until the time of analysis.

Precipitated proteins were digested with trypsin. Tryptic peptides were purified by HPLC chromatography and peptide masses were determined using an LTQ Orbitrap Velos (Thermo Scientific). Proteins were identified by comparing the obtained spectra against the yeast protein sequence database.

6. RESULTS

The major aim of this thesis is to assess the molecular mechanism by which 14-3-3 proteins control DNA replication by regulating components of the replisome at sites of stalled replication. In analogy to Exo1, such factors may also be controlled directly by 14-3-3 proteins and they may play a crucial role in the suppression of replisome restart – the observed phenotype of 14-3-3-deficient strain during HU recovery phase. Our initial analysis focused on the attempt to identify this so called “Factor X” which keeps suppressing the replisome restart of 14-3-3-deficient strain upon HU recovery.

6.1 Analyzing slow fork progression/restart defects linked to Rad53 signaling

Based on the fact that Rad53 deactivation (i.e., dephosphorylation) is required to allow fork restart (Szyjka, Aparicio et al. 2008) and the 14-3-3-deficient cells display persistent Rad53 phosphorylation, we reasoned that it is interesting to test whether a conditional Rad53 inactivation could suppress the replication defects of these cells. This may indeed suggest that "Factor X" does not exist and defective fork restart of 14-3-3-deficient cells upon HU recovery is simply due to continuous signaling caused by the residual activity of Rad53. Thus, we wanted to check whether Rad53 dephosphorylation alone during the HU recovery is sufficient for normal fork restart.

To address this, we set up a tetracyclin-regulatable expression system and overexpressed two phosphatases (namely Glc7 and Pph3) in the 14-3-3-deficient strain, specifically during the HU recovery phase. These two phosphatases were selected according to their established roles in the literature. In fact, Glc7 was shown to promote Rad53 dephosphorylation and recovery from replication fork stalling caused by HU treatment (Bazzi, Mantiero et al. 2010). Similarly, genetic and biochemical evidence suggests that Pph3 forms a complex with Psy2 that binds and dephosphorylates activated Rad53 during recovery from MMS-mediated DNA damage (O'Neill, Szyjka et al. 2007).

Transformed 14-3-3-deficient cells were grown and treated with 150 mM HU in the presence of 1 μg/ml doxycycline, which kept the Tet-system off. Cells were then washed and released into fresh, pre-warmed YPD (without doxycycline) to allow

recovery from the HU block. As seen in figure 17A, 90 minutes after recovery, transformed 14-3-3-deficient cells were able to reduce the level of phosphorylated Rad53 compared to untransformed cells. Although phosphorylated forms of Rad53 never totally disappeared in both conditions, overexpression of two phosphatases resulted in decreased levels of phosphorylated Rad53 at later time points during the HU recovery of 14-3-3-deficient cells.

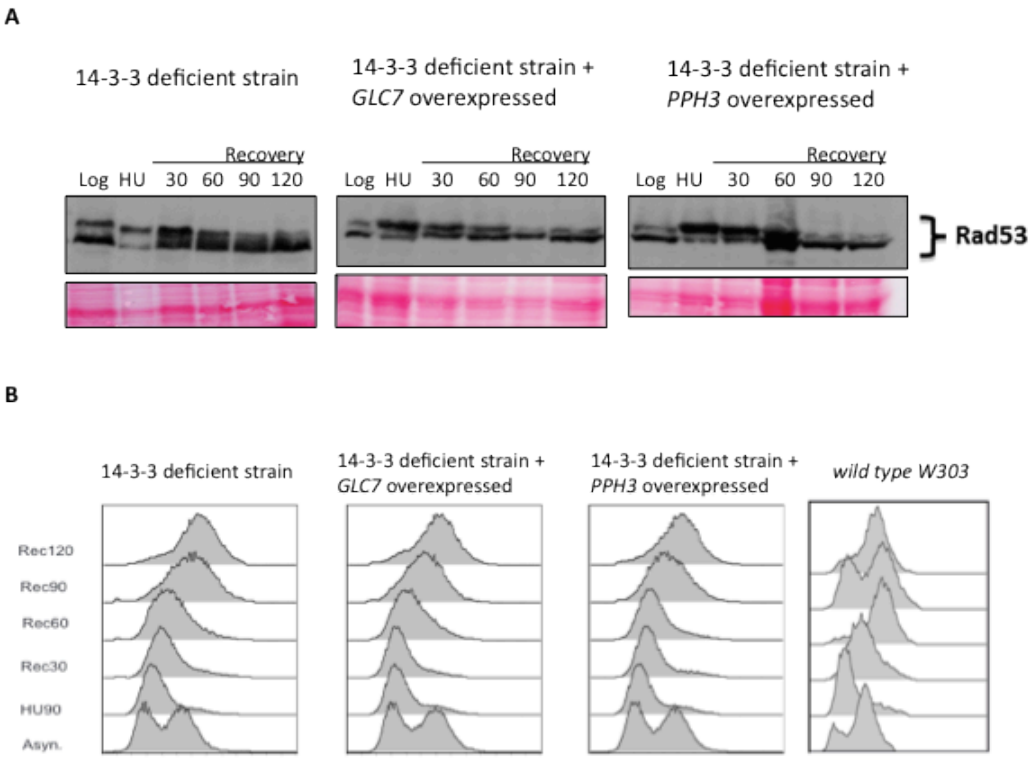


Figure 17: Overexpression of phosphatases *GLC7* and *PPH3* in HU recovery phase of 14-3-3-deficient cells

Log phase cells were treated with 150 mM HU. Cells then washed twice and released into fresh YPD in the absence of doxycycline. Samples were collected at the indicated time points to analyze the phosphorylation-dependent mobility shift of Rad53 by Western blot (A) and the DNA content by FACS (B).

On the other hand, comparison of cell cycle progression during the HU recovery showed no clear difference among the three conditions. 14-3-3-deficient cells expressing either one of the two phosphatases were still very slow in recovering from HU – displaying almost the same kinetics of 14-3-3-deficient cells alone. For

comparison purposes, the cell cycle profile of wild type cells were also added: In this case, the next cell cycle after HU recovery was clearly seen in 90 minutes whereas, irrespective of phosphatase overexpression, 14-3-3-deficient cells could not start the next cell cycle at least 120 minutes after recovery.

6.2 *In silico* analysis of Bmh1 targets

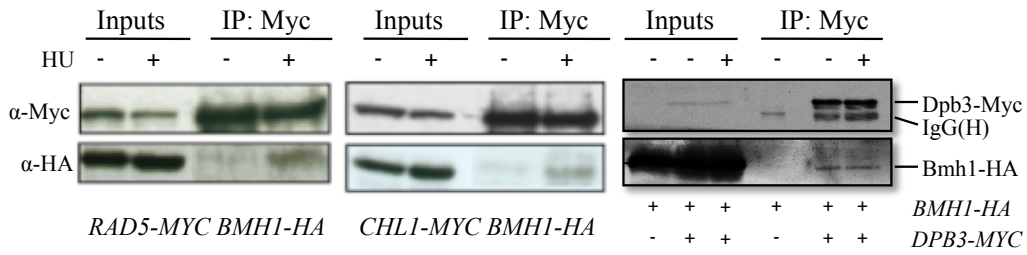
The observation that reduced level of phosphorylated Rad53 did not change the recovery kinetics of 14-3-3-deficient cells further strengthened the possibility that the "Factor X" does exist and suppresses the replisome during HU recovery of 14-3-3-deficient cells. Regarding this, established 14-3-3 interacting proteins were shortlisted by *in silico* selection (www.yeastgenome.org), focusing on those that may have a role in DNA replication/repair events (Table 5). Tagged strains were generated in order to perform immunoprecipitation experiments which were expected to delineate the pattern of Bmh1 interacting partners in basal vs. replication stress conditions.

Gene	**Interaction**	**Annotation**
CAC2 (CAF1)	genetic	Component of chromatin assembly factor
CHL1	genetic	Probable DNA helicase
DPB3	genetic	Third-largest subunit of DNA polymerase II
RAD5	genetic	DNA helicase
RRM3	genetic	DNA helicase
TOF1	genetic	Subunit of a replication-pausing complex

Table 5: *In silico* shortlisted Bmh1 interacting proteins

Our co-immunoprecipitation screen revealed physical interaction between Rad5-Bmh1 and Chl1-Bmh1 proteins in an HU-dependent manner. Besides, Dpb3 and Bmh1 also showed a constitutive interaction, irrespective of replication stress condition (Figure 18A). Other three candidates, shown in figure 18B, did not show any consistent interaction pattern. Indeed, although sometimes relatively weak, bands were observed upon pull-down of Tof1 or Caf1, overall they did not give statistically significant results.

A



B

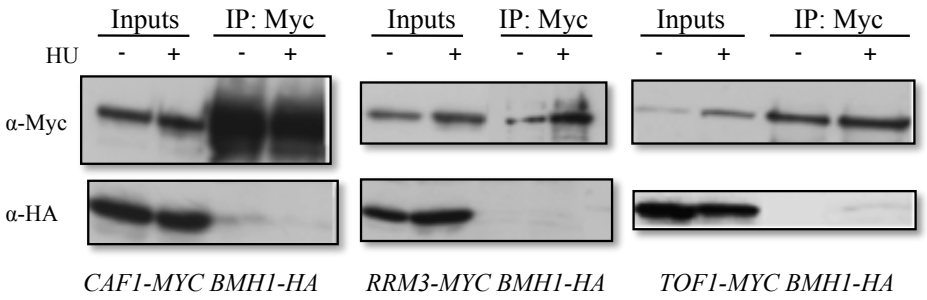


Figure 18: Co-Immunoprecipitation experiments to identify novel physical interactions with Bmh1

Yeast cultures, exponentially growing in YPD, were treated for 90 min with 150 mM HU. Whole cell extracts were immunoprecipitated with the monoclonal antibody to Myc, proteins were resolved on an 8% SDS-polyacrylamide gel and detected as indicated. The experiment for each strain was repeated at least 4 times.

6.3 Identifying novel Bmh1 interacting proteins by mass spectrometry

Our *in silico* analysis identified three promising factors that are potential targets of 14-3-3 proteins. On the other hand, these candidates were selected from a short list of proteins compiled from existing databases. In order to eliminate the possibility that our approach excluded important 14-3-3 targets with a role in the regulation of DNA replication, we decided to perform our own mass spectrometric analysis.

To this end, we first generated the endogenously tagged *BMH1-STREP* strain followed by pull-down studies on whole cell extracts using Strep-Tactin Sepharose beads. Bmh1-Strep was eluted from the beads under mild conditions using D-desthiobiotin, an agonist that binds to Strep-Tactin with high affinity, hence eluting Strep-tagged Bmh1 together with its bound proteins. In order to check for the pull-down, a fraction of the eluates was run on a 10% SDS polyacrylamide gel and proteins were visualized by silver staining. The staining showed that Bmh1-Strep was successfully pulled-down from *BMH1-STREP* cells, but not from negative control wild type cells (Figure 19).

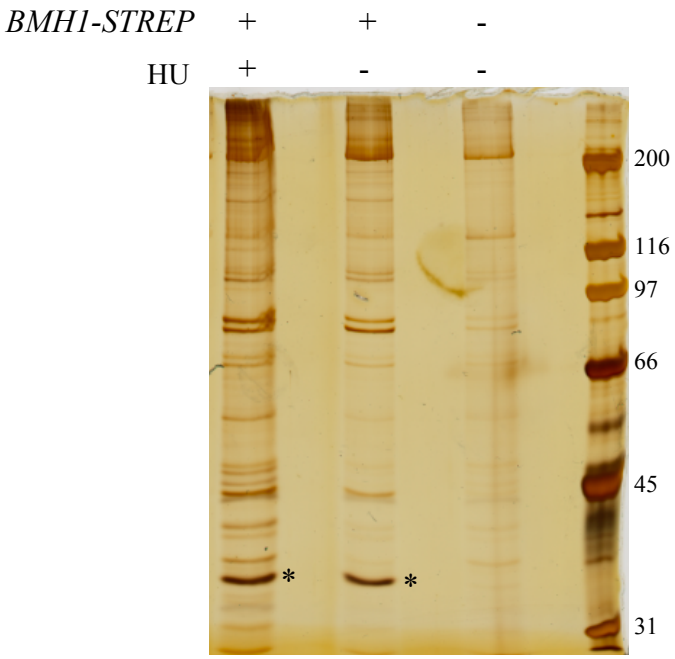


Figure 19: Silver-stained SDS Polyacrylamide gel

Whole cell extracts from exponentially growing wild type and *BMH1-STREP* strains were precipitated with Strep pull-down. For *BMH1-STREP* strain, both untreated and 150 mM HU treated samples were added. Proteins were resolved on a 10% SDS-polyacrylamide gel and silver stained. The asterisk indicates Bmh1-Strep.

Proteins eluted from Strep-Tactin beads were precipitated using TCA as described in Materials and Methods and analyzed by mass spectrometry (MS) using an LTQ Orbitrap Velos (Thermo Scientific). Table 6 shows a selection of the major hits found to interact with Bmh1.

As expected Bmh1 was the top hit, which proves that our set up works (Table 6). Furthermore, the presence of Bmh2 as well as known Bmh1 binding proteins among

the verified hits – namely Reg1, Cyk3 and Rtg2 – confirmed the quality and reliability of the assay.

It was remarkable to see two subunits of type 2A-related serine-threonine phosphatase complex as novel interacting partners of Bmh1. Due to the appearance of the large regulatory subunit Sap190 and the catalytic subunit Sit4, we decided to focus on studying the role of Sit4 as a promising target of replication stress response, instead of concentrating on hits with established roles such as transcription, mitochondrial function, cytokinesis and protein trafficking. Results of studies on Sit4 will be mentioned in chapter 6.8.

Protein	**Mw**	**Function**	**W303 ct**	***BMH1-STREP***	***BMH1-STREP* +HU**
Bmh1	30	Adaptor	0	289	93
Bmh2	31	Adaptor	0	42	13
Sap190	117	Regulatory	0	7	36
Reg1	113	Regulatory	0	29	8
Rpa135	136	Transcription	0	19	11
Ret3	22	Transport	1	18	7
Rpc40	38	Transcription	0	21	4
Por1	30	Mitochondrial	0	0	17
Nsr1	45	Nuclear	1	12	2
Sit4	35	Phosphatase	0	9	6
Cyk3	101	Cytokinesis	0	8	4
Scp160	135	Translation	0	11	1
Rtg2	66	Transcription	0	1	11
Ade16	65	Purine biosynthesis	0	3	3
Mks1	66	RAS pathway	0	4	0
Thr2	40	Aminoacid synthesis	0	0	3
Efb1	50	Translation	73	134	101
Mes1	86	tRNA synthetase	32	341	109
Sec26	109	ER-related	26	145	35
Sec27	99	ER-related	12	15	99
Stm1	30	Suppresor	2	39	21

Table 6: Selection of Bmh1 interacting proteins identified by mass spectrometry

The table shows: hit (column 1), molecular weight (column 2), annotation (column 3) and number of unique peptides from 3 samples; non-tagged control strain (columns 4), *BMH1-STREP* strain untreated (column 5), *BMH1-STREP* strain treated with HU (column 6).

6.4 A novel function of Polε accessory subunit Dpb3 under DNA replication stress

The Polε accessory subunit Dpb3 modulates fork progression under DNA replication stress

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Word count (including references): 5492

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ABSTRACT

Genome stability is ensured by faithful replication of DNA through the activity of polymerases and by surveillance machineries orchestrating repair of damage. We have previously shown that 14-3-3 proteins control Exonuclease-1 (Exo1), avoiding untimely resection at and behind replication forks, thus contributing to replication fork integrity. Our studies indicated that 14-3-3 proteins have an additional role in the control of fork restart. Here we report the identification of Dpb3, one of DNA polymerase epsilon (Polε) accessory subunits, as novel interaction partner of 14-3-3 proteins. We demonstrate that *DPB3* deletion suppresses HU hypersensitivity of 14-3-3-deficient cells and facilitates fork restart. Deletion of *DPB3*, but not deletion of the other accessory subunit *DPB4*, is sufficient to accelerate DNA replication fork progression upon deoxynucleotide depletion by HU. We exclude variations in intracellular dNTP levels as well as a role for translesion polymerases in this effect on DNA replication dynamics. Based on our results and considering that accessory subunits were suggested to stabilize the interaction of Polε with dsDNA, we suggest that lack of Dpb3 may cause a switch from processive to distributive DNA synthesis.

AUTHOR SUMMARY

Stalling and collapse of DNA replication forks is an important source of genome instability and has been implicated in early steps of carcinogenesis. Maintenance of stable intermediates as well as fork restart upon stalled replication requires the coordinated action of a number of proteins. The evidence obtained in the present study indicates that Polε accessory subunit Dpb3, in addition to its established structural role, may restrain DNA synthesis when low levels of deoxynucleotide are available, thus preventing genomic instability in conditions of DNA replication stress.

INTRODUCTION

The fidelity of DNA replication is essential to maintain a stable genome and errors occurring during replication facilitate the development of cancer [1, 2]. In budding yeast, DNA replication starts at defined sequences or origins that are distributed throughout chromosomes and where proteins of the origin recognition complex (ORC) initially bind as cells exit mitosis [3, 4]. Assembly of the MCM complex on ORC licences the origin for initiation, with Cdc45, the GIN complex and the Ctf4 trimer required for subsequent activation of origins. Physical replication of DNA is initiated by polymerase α (Polα) and continuously carried on by polymerase ε (Polε) on the leading strand or discontinuously by polymerase δ (Polδ) on the lagging strand [3, 4].

Similarly to Polα and Polδ, yeast DNA Polε is a multisubunit class B polymerase [5] comprising the catalytic subunit Pol2, which contains the polymerase active site and the domain responsible for proofreading activity, the essential subunit Dpb2 [6] and the accessory subunits Dpb3 and Dpb4 [7, 8]. Dpb3 [9] and Dpb4 [10] are non-essential for cell growth and contain so-called histone-fold motifs homologous to the extended helix-strand-helix structure observed in core histones that is responsible for their dimerization [11]. Along with Dpb2, the Dpb3-Dpb4 dimer helps stabilizing Polε holoenzyme [9, 12] and was proposed to confer Polε the peculiar property of strong affinity for dsDNA [10, 13] as well as to create a platform for interaction with chromatin modifiers [12]. More recently and based on cry-electron microscopy resolution of Polε structure, the extended tail formed by Dpb2-Dpb3-Dpb4 was shown to be flexibly connected to Pol2, with the Dpb3-Dpb4 portion closely interacting with nucleic acids after entry of dsDNA into the active site cleft of the polymerase, in a manner that confers intrinsic processivity to Polε holoenzyme [13]. Biochemical data have confirmed that the Dpb3-Dpb4 dimer confers processive polymerase and 3'-5' exonuclease activity to Polε [14]. In addition, it was also observed that, in the absence of the Dpb3-Dpb4 dimer, Pol2-Dpb2 biochemical properties remain unchanged [12] though the enzyme displays less than 50% of the holoenzyme activity [9], a fact that could be however explained by the decreased stability and, in turn, amounts of polymerase present in deletion mutants [12]. Deletion of *DPB3* showed a modest increase in mutation rate [9], whereas double

deletion of the *DPB3 DPB4* genes led to elevated spontaneous frameshift and base substitution rate *in vivo* [14].

DNA damage represents a physical impediment to replication, causing fork stall followed by collapse, and eventually resulting in chromosome breaks and genome rearrangements [15]. To prevent this, a replication checkpoint has evolved as surveillance mechanism that controls components of the replisome [16], thus allowing to coordinate cell cycle arrest with DNA repair.

Eukaryotic 14-3-3 are highly conserved proteins that establish phosphorylation-dependent interactions modulating protein function in a number of metabolic pathways as well as in protein trafficking, signal transduction, apoptosis and cell-cycle [17]. Structural analysis showed that 14-3-3 proteins self-assemble into flexible homo- and hetero-dimers forming a central groove that is able to adapt two extended peptides [18, 19]. This feature confers them the ability to act as adaptors that integrate signals from different pathways [20, 21]. 14-3-3 proteins can also bind cruciform DNA [22] and replication initiation proteins such as Mcm2 and Orc2 [23]. Upon DNA damage and DNA replication stress, 14-3-3 proteins are required for cell cycle restart, suppression of genomic instability and viability [24]. 14-3-3 proteins genetically and physically interact with the checkpoint protein Rad53 [25] as well as with the acetyltransferases and deacetylases Esa1 and Rpd3 upon replication perturbations [26].

We have previously reported that, under conditions of limiting nucleotide availability, yeast strains deficient for 14-3-3 accumulate ssDNA gaps that cause persistent checkpoint activation and recovery defects [27]. We have also provided evidence that the generation of such gaps at and behind replication forks is Exo1-dependent, whereas the recovery defect is not [27], indicating that Exo1 is only one of the factors targeted by 14-3-3 proteins at stalled forks.

In this study, we identify Dpb3, an accessory subunit of leading strand DNA polymerase epsilon (Polε), as novel interaction partner of the yeast 14-3-3 protein Bmh1. *DPB3* deletion suppresses the HU hypersensitivity of 14-3-3-deficient cells and rescues their ability to restart stalled replication forks. We observe that accelerated fork progression under these conditions is not paralleled by suppression of checkpoint activation, indicating that faster conclusion of S-phase goes at the expenses of quality of replication. We rule out variations in intracellular dNTP levels as well as a role for translesion polymerases in the effect of *DPB3* deletion on DNA

replication dynamics and suggest that this rather allows a switch from processive to distributive DNA synthesis.

RESULTS

DPB3 deletion suppresses DNA damage hypersensitivity of 14-3-3-deficient cells

We previously reported that Exo1 interacts with yeast 14-3-3 proteins which, in turn, restrain Exo1 nucleolytic activity suppressing the accumulation of ssDNA gaps at and behind replication forks [27]. However, we also observed that *EXO1* deletion in 14-3-3-deficient cells does not affect their intrinsic slow DNA replication phenotype [27]. Based on this observation, we set out to identify novel factors that interact with 14-3-3 proteins and may support restart of stalled replication forks. To this end, we conducted an *in silico* analysis of yeast interaction databases [28] and shortlisted candidate targets that satisfy two requirements: (i) genetic interaction with 14-3-3 proteins and (ii) annotated roles in DNA transactions (Supplementary Table 1). Among these, we focused our efforts on Dpb3, one of the two accessory subunits of the leading polymerase Polε.

Since deletion of both 14-3-3 genes (*BMH1* and *BMH2*) is lethal, we have previously chosen a *bmh1-280 bmh2Δ* strain (*bmh* hereafter) [24, 27] for its selective sensitivity and cell cycle recovery defects in response to deoxynucleotide depletion by HU, but otherwise normal growth and cell cycle progression in unperturbed conditions [24]. To assess the effect of *DPB3* deletion on the sensitivity of *bmh* cells to HU we performed viability assays. We observed that, in comparison to wild type, single deletion of *DPB3* did not affect colony formation at increasing HU doses. On the other hand, *DPB3* deletion in the *bmh* background rescued the HU-hypersensitivity of 14-3-3-deficient cells (Fig. 1A). Given the slightly smaller size of colonies formed by *dpb3Δ* cells as compared to wild type cells (Fig. 1A), possibly indicative of differences in growth rate, to more precisely assess the rescue of HU-hypersensitivity we examined exponential cell growth in liquid culture. Experiments conducted in medium containing 20 mM HU showed that logarithmic cultures of wild type and *dpb3Δ* strains displayed similar growth rates, whereas the slower growth rate of *bmh* cells was slightly increased upon *DPB3* deletion (Fig. 1B). Hence, these data support the conclusions drawn from the colony formation assays.

To examine whether rescue of HU-hypersensitivity and slow cell cycle progression in HU would be phenocopied by deletion of *DPB4*, we deleted the gene coding for the second accessory subunit of Polε that forms a dimer with Dpb3. *Dpb3Δ* and *dpb4Δ* strains showed similar sensitivity to HU (Fig. 1A). However, *DPB4* deletion in the

bmh background did not rescue the HU-hypersensitivity of 14-3-3-deficient cells (Fig. 1A).

These data indicate that *DPB3* deletion causes partial suppression of the replication defects of 14-3-3-deficient cells, and that this effect is independent on the second accessory subunits of Polε.

DPB3 deletion facilitates recovery from HU arrest

To assess whether the rescue of HU-hypersensitivity observed in the *bmh dpb3Δ* strain results from effects on replication, we compared cell cycle progression in single and double mutants, using *dpb4Δ* cells as control.

Flow cytometric analysis of the DNA content in cells recovering from HU treatment showed that *DPB3* deletion accelerated S-phase progression of 14-3-3-deficient cells (Fig. 2A, 30 to 60 min time-points). On the other hand, deletion of the second accessory subunits of Polε, *DPB4*, had no effect on the slow rate of S-phase progression of 14-3-3-deficient cells (Fig. 2A).

To examine the status of the replication checkpoint under these conditions, we performed Western blot analysis of Rad53. The pattern of sustained checkpoint activation in the *bmh* strain [24], evidenced by delayed and incomplete Rad53 dephosphorylation upon release from HU [27], was substantially similar at early time points in the double mutant and became more marked at late time points in these cells, in comparison to the pattern of the *bmh* strain (Fig. 2B). Flow cytometry data confirmed that *bmh dpb3Δ* cells were still in G2/M at 90 min from the time of release, while wild type or *dpb3Δ* single mutant cells already entered the next cell cycle at this time point (Fig. 2A).

Taken together, these data show that *DPB3* deletion facilitates replication restart after nucleotide depletion in 14-3-3-deficient cells, but fails to suppress the prolonged checkpoint activation observed in this genetic background, suggesting that faster conclusion of S-phase may occur at the expenses of the quality of replication.

Dpb3 physically interacts with Bmh1

To assess whether Dpb3 and yeast 14-3-3 proteins are able to form a complex, we performed protein interaction studies. Pull-down experiments showed that Strep-tagged Dpb3 physically interacts with Bmh1 both in control and HU-treated cells

(Fig. 3A). To complement this observation using a distinct protocol, we immunoprecipitated Myc-tagged Dpb3 and revealed HA-tagged Bmh1 by immunoblotting. The results confirmed physical interaction of the two proteins (Fig. 3B).

Hence, in addition to the genetic interaction that was previously described [28], these data show that Bmh1 and Dpb3 physically interact in *S. cerevisiae* cells.

DPB3 deletion increases the rate of fork progression of 14-3-3-deficient cells

To assess whether the rescue of both HU-hypersensitivity and slow S-phase progression in the *bmh dpb3Δ* strain results from effects on replication, we performed neutral-neutral bi-dimensional (2D) gel electrophoresis upon HU arrest and release on the early origin of replication ARS305 (Fig. 4A), which is activated in response to HU [29]. In wild type and *dpb3Δ* cells, typical replication intermediates (RIs) accumulated on ARS305 during the arrest and were rapidly restarted - clearing the origin fragment - 60min after drug removal (Fig. 4B), proving efficient replication restart. At 90min, faint RIs mark a second, asynchronous replication round after HU release (Fig. 4B; see also Fig. 2A). According to our previous report [27], the pattern of RIs observed in *bmh* cells undergoing HU block/release indicates an extremely slow progression of replication forks during recovery, with RIs still close to the origin 60 min after HU removal (Fig. 4B). Deletion of *DPB3* in the *bmh* background resulted in an RI pattern similar to that of wild type cells, suggesting that lack of the accessory subunit of Polε rescues the fork-restart defect of 14-3-3-deficient cells upon release from HU.

To better appreciate the effects of *DPB3* deletion on replication fork rate under deoxynucleotide shortage, we used 2D gel analysis to monitor RI appearance and clearance on ARS305 and on adjacent fragments (Fig. 5A and 5B), in cells synchronously released from G1 in 200 mM HU. Also under these conditions, we obtained evidence that *DPB3* deletion in the *bmh* background leads to faster fork progression, evidenced by an earlier clearance of the ARS305 fragment and accelerated transit of forks into fragments A and B (Fig. 5B, 30 min; Fig. 5C, 90-120 min). Faster fork progression upon HU treatment likely explains also the lower intensity of the bubble signal observed in *dpb3Δ* cells upon HU arrest of asynchronous cells (Fig. 4B). Thus, *DPB3* deletion allows more extensive DNA

synthesis upon conditions of marked deoxynucleotide shortage and suppresses defective restart of HU-arrested forks in *bmh* cells.

To further assess how Dpb3 controls DNA synthesis upon limiting deoxynucleotide levels, we synchronously released wild type and *dpb3Δ* cells into a low HU dose, a treatment that allows following S-phase transition and completion by FACS analysis of DNA content [26]. Time-course analysis of cells released in 20 mM HU showed that single *DPB3* deletion was effectively causing faster transition through S-phase when compared with wild type cells (Fig. 6A, 45 min onwards), a response that was accompanied by delayed checkpoint kinase activation (Fig. 6B).

Taken together, these data show that deletion of the Polε accessory subunit Dpb3 generally promotes faster S-phase transition under conditions of limited dNTP availability and facilitates fork restart in the *bmh* background.

DPB3 deletion may affect the DNA synthesis mode

Since it was shown that DNA replication mutants may affect replication dynamics through changes in intracellular dNTP levels [30], we asked whether the acceleration of S-phase progression observed in our studies would be merely the consequence of effects on the dNTP pool resulting from *DPB3* deletion. Quantifications performed from extracts of wild type or *dpb3Δ* cells treated with HU did not reveal significant variations of the dNTP pool (Fig. 7A), ruling out this as possible explanation of the effect of *DPB3* deletion.

We reasoned that another possible cause of the acceleration of S-phase progression observed in the *dpb3Δ* background could be the recruitment of translesion polymerases at stalled forks in place of the replicative leading strand polymerase [31]. To test this hypothesis, we deleted *DPB3* in the translesion-null background (*TLSΔ*) of the *rev7Δ, rev3Δ, rev1Δ, rad30Δ* strain [32]. Colony formation assays and flow cytometric analysis showed that wild type, single- and double-mutant cells displayed similar resistance to HU (Fig. 7B) and similar pattern of cell cycle progression (Fig. 7C). These data rule out a role for translesion polymerases in the fork acceleration effect observed upon *DPB3* deletion.

Based on data available in the literature on the structure of Polε [13], a third possible explanation for the phenotype observed upon deletion of *DPB3* would be a switch of the polymerase from a processive to a more distributive mode of action, possibly

associated with repetitive re-priming events using residual available deoxynucleotides. Such a scenario, which was suggested to explain biochemical evidence indicating that Dpb3 and Dpb4 act by stabilizing the Polε holoenzyme on DNA [14], is expected to lead to ssDNA gap accumulation on replicating duplexes [33], possibly detectable by direct inspection of RIs [34]. To accurately survey the effects of *DPB3* deletion on replicating DNA, we thus visualized RIs by psoralen crosslinking coupled to electron microscopy, an approach that has the potential to reveal ssDNA gaps ≥ 30-40nt long [35]. The data showed that the number of molecules displaying detectable ssDNA gaps in bubbles and forks was similar in *WT* and *dpb3Δ* cells (Fig. 8). Furthermore, no significant differences were observed when the size of ssDNA gaps (Fig. S1A) or the size of ssDNA at junctions in bubbles (Fig. S1B) or forks (Fig. S1C) were examined. The subtle increase observed in *dpb3Δ* cells in gap abundance (Fig. 8, Y/dY) and in the length of ssDNA regions at the junction (Fig. S1, B-C) is non-significant for the number of molecules that can be reasonably analyzed by this time-consuming approach. It is possible however, that these subtle differences are affected by the detection limit of the technique (ssDNA gaps ≤ 30-40nt cannot be unambiguously detected) and by the limited number of molecules that can be analyzed.

Taken together these data show that neither variation in the levels of dNTPs nor replacement of the leading-strand DNA polymerase by translesion polymerases is the cause of accelerated S-phase progression in *DPB3* deleted cells. Although our EM data were not revealing in this respect, our observations leave open the possibility that frequent repriming by DNA Polε - implicated by biochemical investigations on Dpb3 [14] - allows the continued DNA synthesis and accelerated S-phase transition observed in *dpb3Δ* cells upon deoxynucleotide shortage.

DISCUSSION

We have previously reported that 14-3-3-deficient cells tend to accumulate ssDNA gaps at and behind replication forks, resulting from the unrestrained nuclease activity of Exo1 [27]. The latter is a key component of DNA repair pathways [32, 36-40], but may turn to pathological processor of gaps that are not timely addressed by DNA repair machineries [41, 42]. Here we have addressed another peculiar aspect of 14-3-3-deficient cells, namely their defective replication fork restart and fork progression, under conditions of deoxynucleotide depletion. We report the product of the *DPB3* gene, an accessory subunit of the leading DNA Polε, as physical interacting partner of Bmh1 and we describe the role of Dpb3 in the peculiar phenotype of 14-3-3-deficient cells.

In the course of our studies we observed that *DPB3* deletion suppresses the HU hypersensitivity of 14-3-3-deficient cells and rescues their ability to restart stalled replication forks. Slight acceleration of fork progression was also observed upon single *DPB3* deletion in wild type cells, though this effect was more marked in the slow S-phase phenotype of *bmh* cells. Interestingly, deletion of *DPB4* could not phenocopy the effect of *DPB3* deletion, although biochemical data indicate that Dpb3 and Dpb4 purify as a heterodimeric complex thanks to their histone-fold motifs [10, 12]. However, whereas in the absence of Dpb3, the Dpb4 subunit is not recruited to the Pol2-Dpb2 complex [9], in *dpb4Δ* cells the Pol2-Dpb2-Dpb3 complex was shown to assemble, though in a less stable form than in wild type cells [12]. Additionally, whereas Dpb3 is an exclusive subunit of Polε, Dpb4 also forms complexes with Dls1 as part of the ISW2 chromatin remodeling complex, acting as anchor point for the ISW2 during nucleosome mobilization [43]. Hence, evidence available in the literature on the slightly different roles of *DPB3* and *DPB4* supports and helps explaining the distinct phenotype observed in our studies.

We observed that, despite an apparent faster completion of S-phase, *bmh dpb3Δ* cells displayed sustained activation of the checkpoint kinase Rad53, suggesting that accelerated progression through S-phase may have occurred at the expenses of the quality of replication. Data available in the literature indeed show that deletion of *DPB3* and *DPB4* elevates mutation rates to a level comparable to that observed in a proofreading deficient *pol2* strain, suggesting an influence of the two genes on the fidelity of replication [14]. Hence, one is tempted to speculate that a possible role for

Dpb3 in wild type cells, in addition to its established structural functions [7, 8, 13], is to restrain DNA synthesis under conditions of low deoxynucleotide availability.

With regard to the molecular mechanism underlying facilitated S-phase progression upon *DPB3* deletion, we examined different possibilities. First, an increase in intracellular dNTP levels was described to facilitate replication of DNA templates under replication stress [30]. Quantifications of the dNTP pool in wild type or *dpb3Δ* cells treated with HU did not reveal significant variations, ruling out this as possible explanation for the phenotype observed. Second, evidence available in the literature indicates that translesion synthesis polymerases participate in replication of undamaged DNA when synthesis by replicative polymersases is defective [31]. HU-sensitivity and S-phase progression of cells where translation polymerases were deleted in the d*pb3Δ* background showed a pattern similar to wild type cells, ruling also out a role for translesion polymerases as reason for the experimental evidence. Third, based on the high affinity for dsDNA conferred to Polε by the Dpb3-Dpb4 dimer [10, 13], we reasoned that the acceleration of replication forks in *dpb3Δ* cells might result from loose binding of Polε to DNA, leading to frequent re-priming events. This possible mode of action of Dpb3-deficient Polε is substantiated by structural data [13] and was suggested by Aksenova and collaborators to explain the *in vitro* polymerase and exonuclease activities of Pol2 in the absence of its accessory subunits [14]. To test this hypothesis, we examined whether *dpb3Δ* cells tend to accumulate more ssDNA gaps than wild type cells and we determined their extent and size using electron microscopy. Our data did not provide a conclusive answer to this question, possibly due to the limits of this EM approach in visualizing ssDNA gaps ≤ 30-40nt. Hence, the possibility that lack of the Dpb3 accessory subunit may cause frequent repriming by Pol2, switching its mode of action from processive to distributive, remains open and will be the subject of intense future studies in our laboratory.

MATERIALS AND METHODS

Materials

The antibodies used in this study were: mouse monoclonals anti-HA (12CA5, Sigma); mouse monoclonal anti-Myc (9E10, Santa Cruz Biotechnology) and goat polyclonal anti-Rad53 (yC-19, Santa Cruz Biotechnology).

The chemicals and peptides used in this study were: Hydroxyurea (Bio Basic); a1-Mating Factor (Primm, Milan, Italy). Affinity-based protein pull-down was performed with *Strep*-Tactin® Sepharose beads (IBA Life Sciences).

Saccharomyces cerevisiae strains

Yeast strains used in this study are isogenic to W303-1A (*wild type*) [44] and are listed in Table 1.

Cell synchronization and flow cytometry

Yeast strains were grown in YPD medium (1% yeast extract, 2% peptone, 2% glucose). For block-release experiments, exponentially growing cells grown at 28°C were synchronized in G1 with 3 μg/ml α-factor and released by adding 20 mg/ml Pronase E (Sigma).

The DNA content of individual cells was measured using a CyAn ADP cell sorter. Cells fixed in 70% ethanol were resuspended in 50 mM Tris buffer (pH 7.5), treated with 1 mg/ml RNAase over night at 37°C and DNA was stained with propidium iodide (Molecular Probes).

HU sensitivity assays

Spot dilution assays were performed by plating 6 μl of serial 10-fold dilutions of a liquid culture containing 200 – $2x10^6$ cells/ml. Cells were spotted on YPD plates containing different HU concentrations and grown for 3 days.

Protein extraction, Western Blotting, immunoprecipitation and pull-down assays

Western blot analysis of yeast proteins was carried out upon TCA extraction [45] as previously described [27].

For immunoprecipitation yeast proteins were extracted using ice-cold lysis buffer (25 mM Tris-HCl pH 7.4, 15 mM NaCl, 15 mM EGTA, 1mM NaF, 1mM Na

orthovanadate, 4 mM p-Nitro-Phenyl-Phosphate (pNPP), 0.1% Triton X-100, 1mM PMSF, complete protease inhibitors cocktail (Roche)). Dpb3-Myc was immunoprecipitated using the monoclonal antibody 9E10 to the Myc-tag. Dpb3-Strep was pulled down from 5 mg total cell extracts using *Strep*-Tactin® Sepharose beads.

2D gel electrophoresis and electron microscopy

DNA extraction with the CTAB method and neutral-neutral two-dimensional gel electrophoresis were performed as described [46]. EM analysis [47] was performed as described.

Determination of dNTP levels

Strains were grown in YPAD media (1% yeast extract, 2% bacto peptone, 0.002% adenine, 2% dextrose) at 30°C. Approximately 3.7×10^8 cells (determined by OD_{600}) were harvested by filtration through 25 mm AAWP nitrocellulose filters (0.8 µm; Millipore AB, Sweden). The filters were immersed in 700 µl of ice-cold extraction solution (12% [w/v] trichloroacetic acid, 15 mM $MgCl_2$) and frozen in liquid N_2. The following steps were carried out at 4°C. The tubes were vortexed for 30 sec and incubated for 15 min on a shaker. Filters were removed, and the 700 µl supernatants were collected after centrifugation at 20,000 × *g* for 1 min and added to 800 µl of ice-cold Freon-trioctylamine mixture [10 ml of Freon (1,1,2-trichloro-1,2,2-trifluoroethane, Sigma-Aldrich Sweden AB, > 99%) and 2.8 ml of trioctylamine, Sigma-Aldrich Sweden AB, 98%]. Samples were vortexed and centrifuged for 1 min at 20,000 × *g*. The aqueous phase was collected and added to 700 µl of ice-cold Freon-trioctylamine mixture and vortexed and centrifuged for 1 min at 20,000 × *g*. Volumes of 475 and 47.5 µl of the aqueous phase were collected. The 475 µl aliquots of the aqueous phase were adjusted with 1 M ammonium carbonate ($(NH_4)_2CO_3$) to pH 8.9, loaded on boronate columns (Affi-Gel Boronate Gel, Bio-Rad), and eluted with 50 mM $(NH_4)_2CO_3$, pH 8.9, 15 mM $MgCl_2$ to separate dNTPs and NTPs. The eluates (purified dNTPs) were adjusted to pH 3.4 with 6 M HCl, separated on a Partisphere SAX HPLC column (125 mm x 4.6 mm, Hichrome, United Kingdom), under isocratic elusion with 0.35 M potassium phosphate buffer (pH 3.4; containing 2.5% [v/v] acetonitrile) and quantified using a LaChrom Elite® HPLC system (Hitachi). The 47.5-µl aliquots of the aqueous phase were adjusted to pH 3.4 and used to quantify NTPs by HPLC in the same way.

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FIGURE LEGENDS

Figure 1 – *DPB3* deletion rescues HU-hypersensitivity of 14-3-3 deficient yeast cells

A. *Wild type (WT), bmh1-280 bmh2Δ (bmh), dpb3Δ, bmh1-280 bmh2Δ dpb3Δ (bmh dpb3Δ), dpb4Δ and bmh1-280 bmh2Δ dpb4Δ (bmh dpb4Δ)* cultures were exponentially grown. Serial dilutions (1:10) were spotted on YPD plates containing different HU concentrations and grown for 3 days before scoring.

B. The strains described in A were exponentially grown in full medium (YPD) or YPD containing 20 mM HU. Aliquots were withdrawn at the indicated times and OD_{600} was measured. Values are the average of three independent experiments.

Figure 2 – Deletion of *DPB3,* but not *DBP4*, rescues the slow S-phase progression phenotype of 14-3-3 deficient cells

A. Time-course flow cytometric analysis of the DNA content in the indicated strains upon recovery from a HU-arrest.

B. Western blot analysis showing the phosphorylation-dependent mobility shift of Rad53 during the HU-arrest and the recovery phase in the indicated strains.

Figure 3 - Dpb3 physically interacts with Bmh1

A. Yeast cultures expressing Bmh1-HA and Dpb3-Strep as indicated, were treated for 90 minutes in the presence or the absence of HU (150 mM). Whole cell extracts (WCE, 5mg) were submitted to pull-down studies using Strep-Tactin beads, proteins were resolved on an 10% SDS-polyacrylamide gel and detected as indicated. Input = 100 μg WCE. PR = Ponceau Red.

B. WCE (10mg) from yeast cultures expressing Bmh1-HA and Dpb3-Myc as indicated were immunoprecipitated with a monoclonal antibody to the Myc-tag and examined as described in A.

Figure 4 - *DPB3* deletion facilitates recovery of 14-3-3 deficient cells from HU arrest

A. Schematic representation of RIs visualized by 2D gel electrophoresis.

B. Representative 2D gels of replication intermediates (RIs) at the replication origin ARS305 analyzed after 90 min HU-treatment and upon HU removal (recovery, 60 and 90 min).

Figure 5 - 2D gel analysis of RIs from wild type and mutant strains

A. Chromosome III region adjacent to ARS305 with indication of restriction sites (E= *EcoRV*; H= *HindIII*) and probes (ARS305, A and B) used in 2D gel analysis.

B. Time-course resolution of RIs obtained from the indicated strains that were arrested in G1 (a-factor) and released in S-phase in the presence of 0.2 M HU. Genomic DNA was extracted at the indicated time points, digested and resolved as described in Materials and Methods. RIs were revealed with probes to ARS305 and region A.

C. The membranes shown in B were stripped and RIs revealed with a probe to region B.

Figure 6 – Single *DPB3* deletion accelerates S-phase progression

A. Time-course flow cytometric analysis of the DNA content for the indicated strains upon synchronization in α-factor and release in S-phase in the presence of 20 mM HU.

B. Western blot analysis of Rad53 in cells treated as in A.

Figure 7 – *DPB3* deletion does not affect the dNTPs pool and translesion polymerases do not complement the lack of Dpb3

A. dNTP levels were determined as described in Materials and Methods in the strains indicated upon treatment with HU (150 mM, 90 min).

B. Cultures of the indicated strains were exponentially grown. Serial dilutions (1:10) were spotted on YPD plates containing different HU concentrations and grown for 3 days before scoring. The *bmh* strain was used as control.

C. Time-course flow cytometric analysis of the DNA content for the strains described in B upon recovery from α-factor arrest.

Figure 8 – Quantification of ssDNA gaps in wild type and *dpb3Δ* cells.

Graphical distribution of ssDNA gap number. The number of molecules containing gaps vs. the total number of molecules analyzed in bubbles and forks (Y/dY) is indicated in brackets.

Table 1 - List of *S. cerevisiae* strains used in this study

Strain Name	Genotype	Reference
W303-1A	*MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 [phi+]*	39
YKE2	*MATa bmh2Δ::NAT1 bmh1Δ::HIS3::bmh1-280::LEU2*	24
YKE19	*MATa dpb3Δ::KANMX4*	This study
YKE21	*MATa bmh2Δ::NAT1 bmh1Δ::HIS3::bmh1-280::LEU2 dpb3Δ::KANMX4*	This study
YKE39	*MATa BMH1-HA::URA3::bmh1*	24
YKE23	*MATa BMH1-HA::URA3::bmh1 DPB3-Myc::KANMX4::dpb3*	This study
YMA118	*MATa BMH1-HA::URA3::bmh1 DPB3-STREP::KANMX4::dpb3*	This study
YMA107	*MATa dpb4Δ::KANMX4*	This study
YMA108	*MATa bmh2Δ::NAT1 bmh1Δ::HIS3::bmh1-280::LEU2 dpb4Δ::KANMX4*	This study
YKE78	*MATa RAD5+ rev7Δ::HIS3 rev3Δ::TRP1 rev1Δ::KANMX6 rad30Δ::KANMX6*	30
YMA134	*MATa RAD5+ rev7Δ::HIS3 rev3Δ::TRP1 rev1Δ::KANMX6 rad30Δ::KANMX6 dpb3Δ::URA3*	This study

SUPPLEMENTARY MATERIAL

Supplementary Table 1 - Candidate targets of the yeast 14-3-3 gene *BMH1* with roles in DNA transactions that were shortlisted after *in silico* analysis of yeast interaction databases [1]. The established type of interaction is indicated: G = genetic interaction; P = physical interaction.

gene	interaction	annotation
ARP9	G	Component of SWI/SNF and RSC chromatin remodeling complexes
CAC2	G	Component of chromatin assembly complex
CDC6	G	Required for DNA replication
CDC13	G	Single stranded DNA-binding protein / telomere capping
CHL1	G	Required to establish sister-chromatid pairing during S-phase
DPB3	G	Third-largest subunit of DNA polymerase II
EXO1	P	5'-3' exonuclease and flap-endonuclease
MEC3	G	Subunit of a heterotrimeric complex (Rad17p-Mec3p-Ddc1p)
ORC1	P	Largest subunit of the origin recognition complex
POL1	G	Catalytic subunit of the DNA polymerase I alpha-primase complex
POL12	G	B subunit of DNA polymerase alpha-primase complex
PRI1	G	Subunit of DNA primase
PRI2	G	Subunit of DNA primase
RAD5	G	DNA helicase
RAD9	G	DNA damage-dependent checkpoint protein
REV3	G	Catalytic subunit of DNA polymerase zeta
RFA1	G	Subunit of heterotrimeric Replication Protein A (RPA)
RFA2	G	Subunit of heterotrimeric Replication Protein A (RPA)
RFC1	G	Subunit of heteropentameric Replication factor C (RF-C), clamp loader
RLF2	G	Largest subunit of the Chromatin Assembly Complex (CAF-1)
RRM3	G	DNA helicase
XRS2	G	DNA repair protein component of the Mre11 complex

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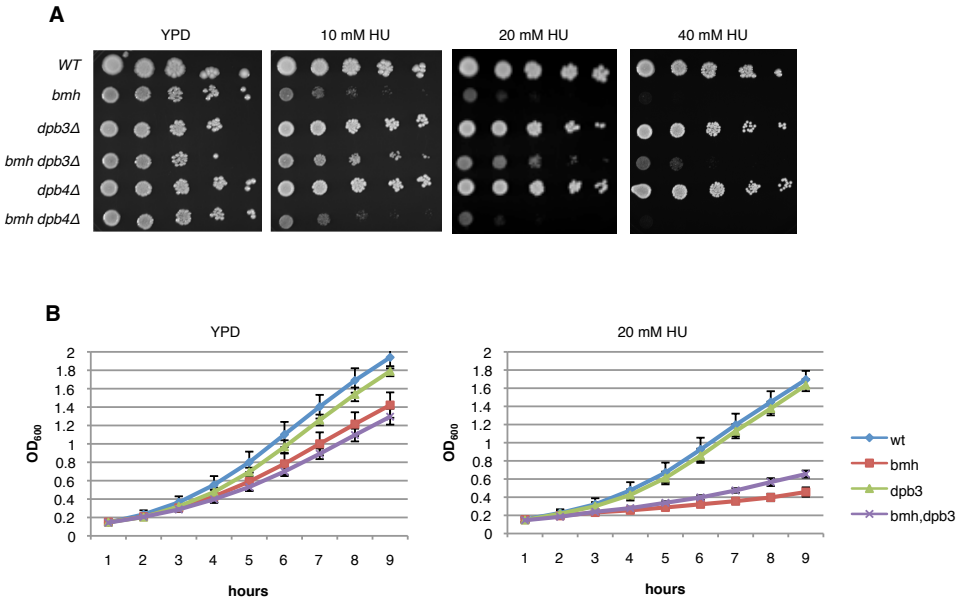
SUPPLEMENTARY FIGURE LEGENDS

Figure S1 – Quantification of ssDNA gap size at bubbles and forks

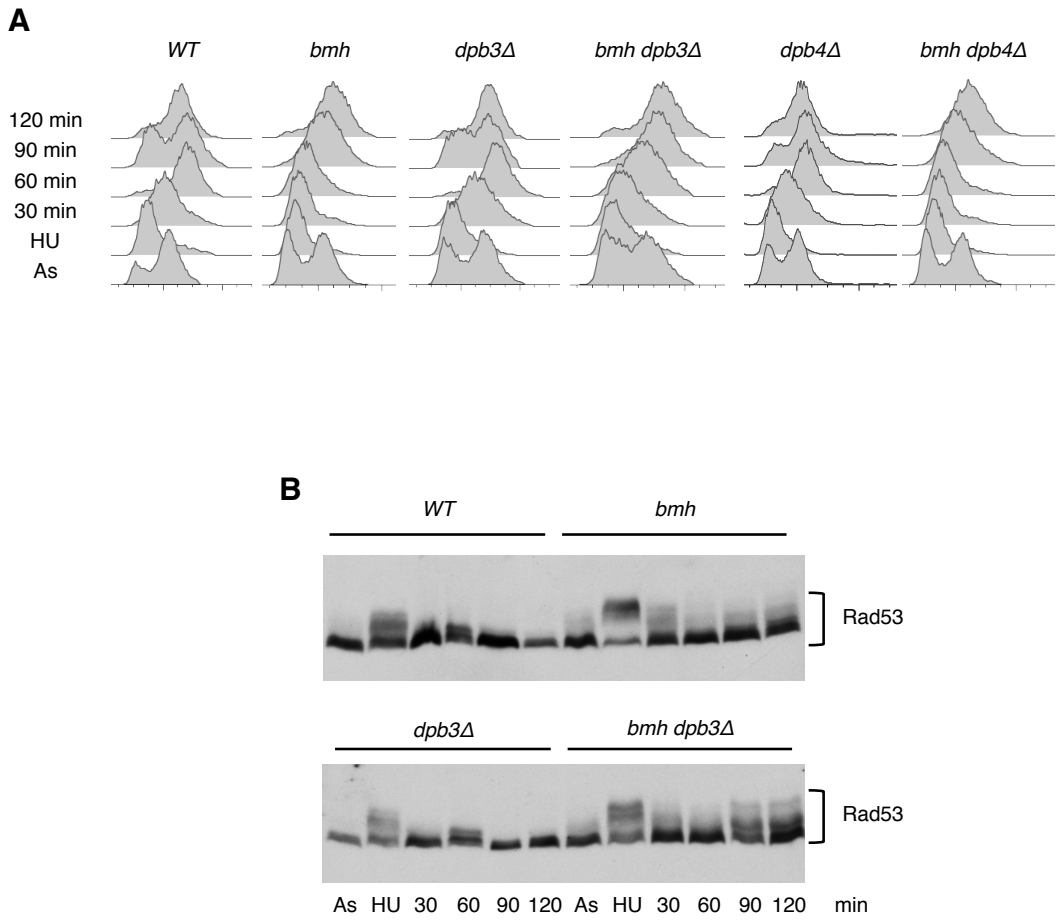
A. Graphical distribution of ssDNA gap size along replicated duplexes. The median of ssDNA gap size in specific sets of analyzed molecules is shown. In brackets, the number of molecules with gaps vs. the total number of molecules analyzed is indicated.

B. Graphical distribution of ssDNA length at the junction in bubbles. Only molecules with detectable ssDNA stretches are included in the analysis. The median length of the ssDNA region at the fork is shown. In brackets, the number of molecules with ssDNA at the junction vs. the total number of molecules analyzed is indicated.

C. Graphical distribution of ssDNA length at the junction in forks. Only molecules with detectable ssDNA stretches are included in the analysis. The median length of the ssDNA region at the fork is shown. In brackets, the number of molecules with ssDNA at the junction vs. the total number of molecules analyzed is indicated.



Aykut et al. Fig. 1



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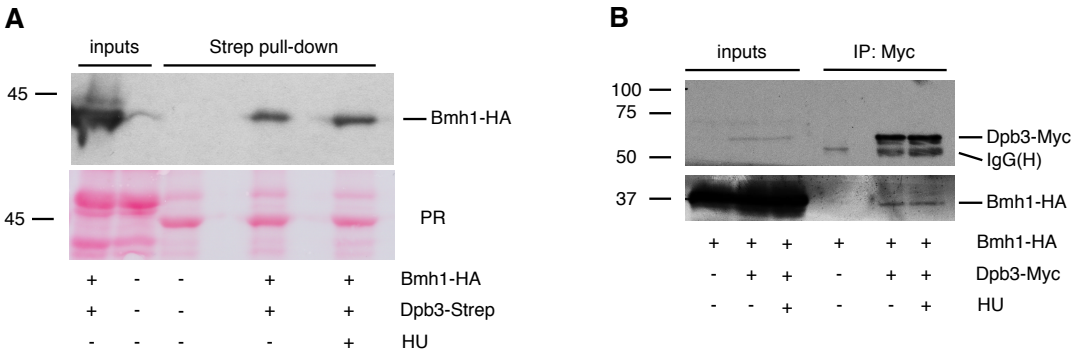

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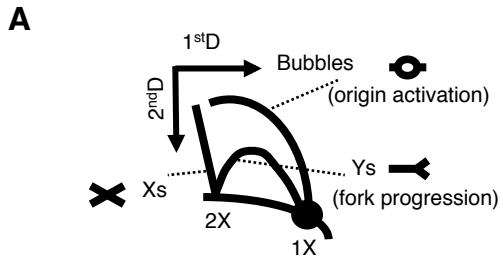

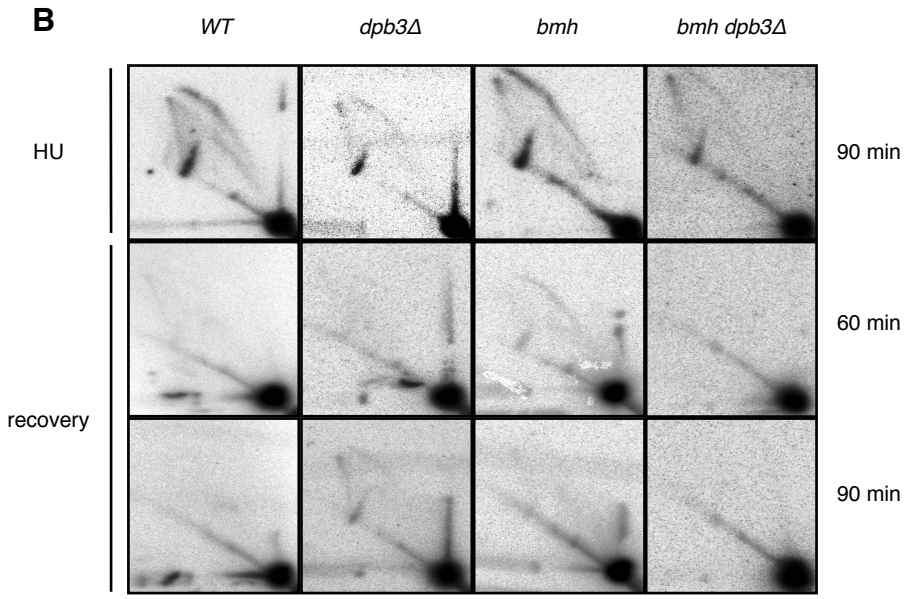

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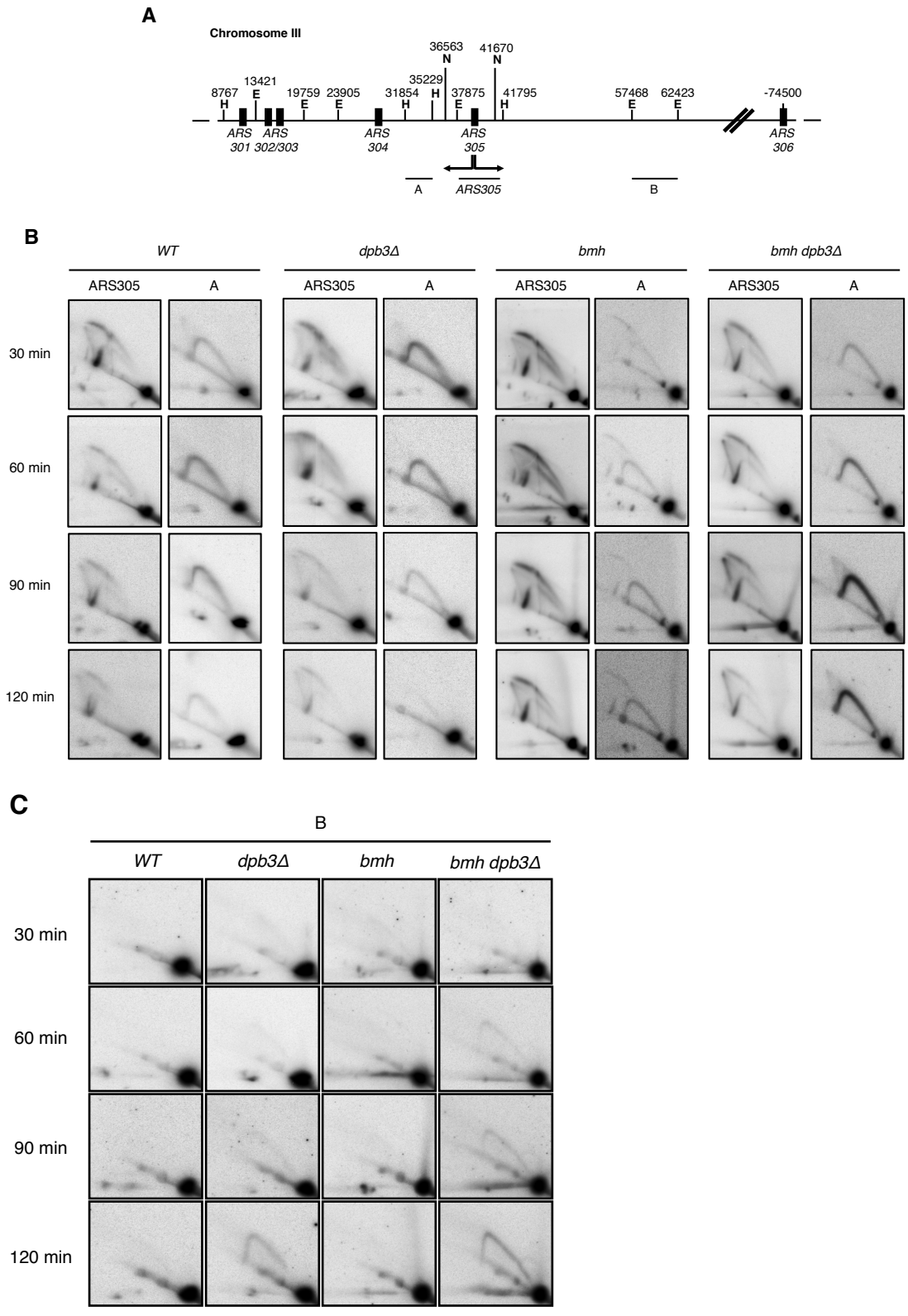
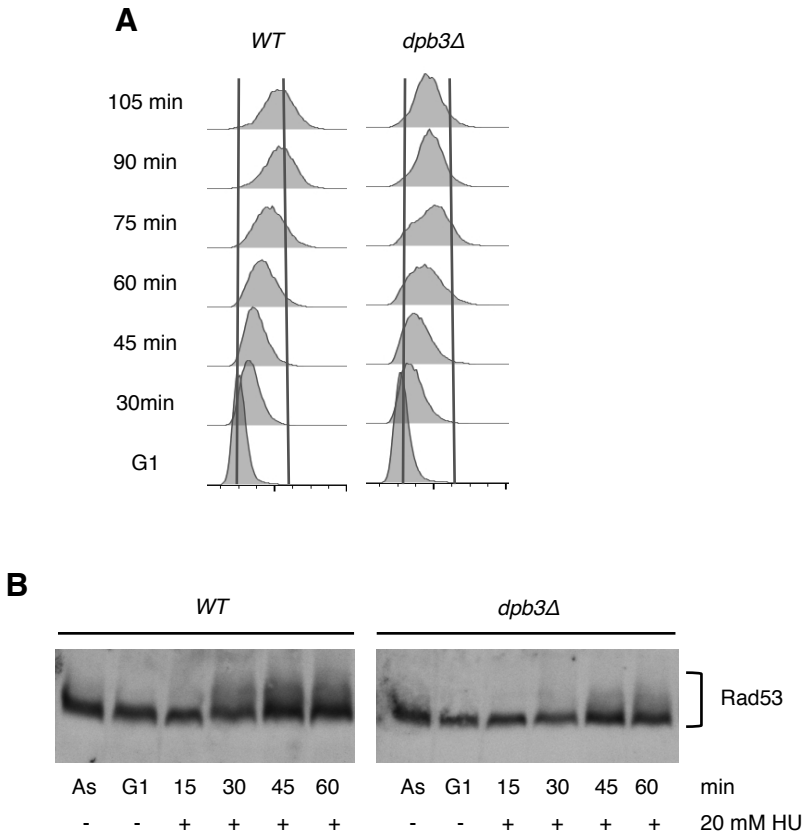
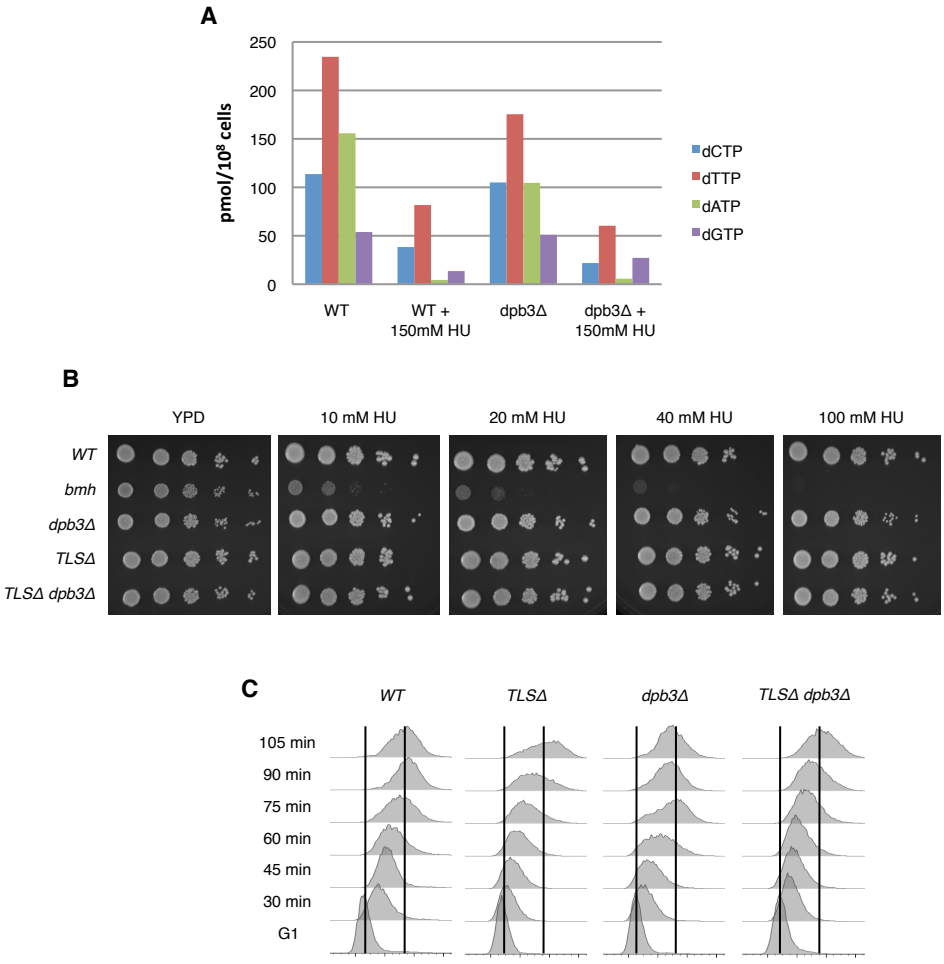


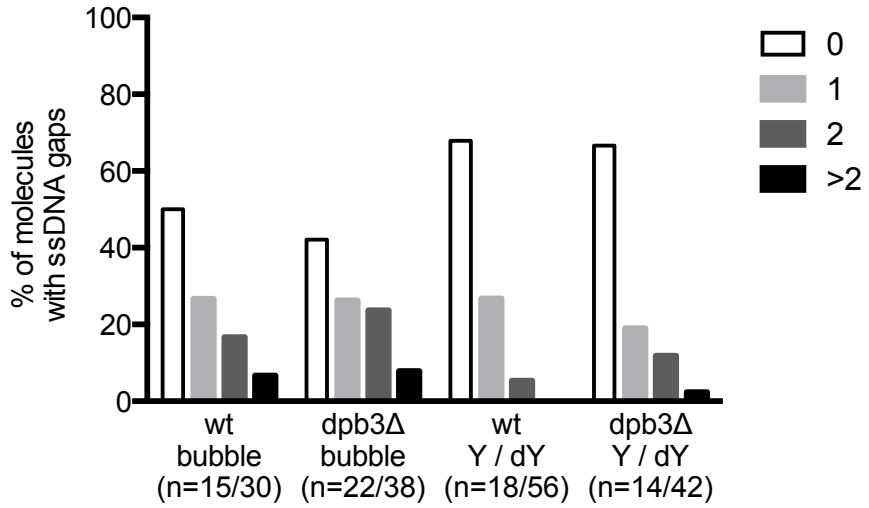
Aykut et al. Fig. 5



Aykut et al. Fig. 6



Aykut et al. Fig. 7



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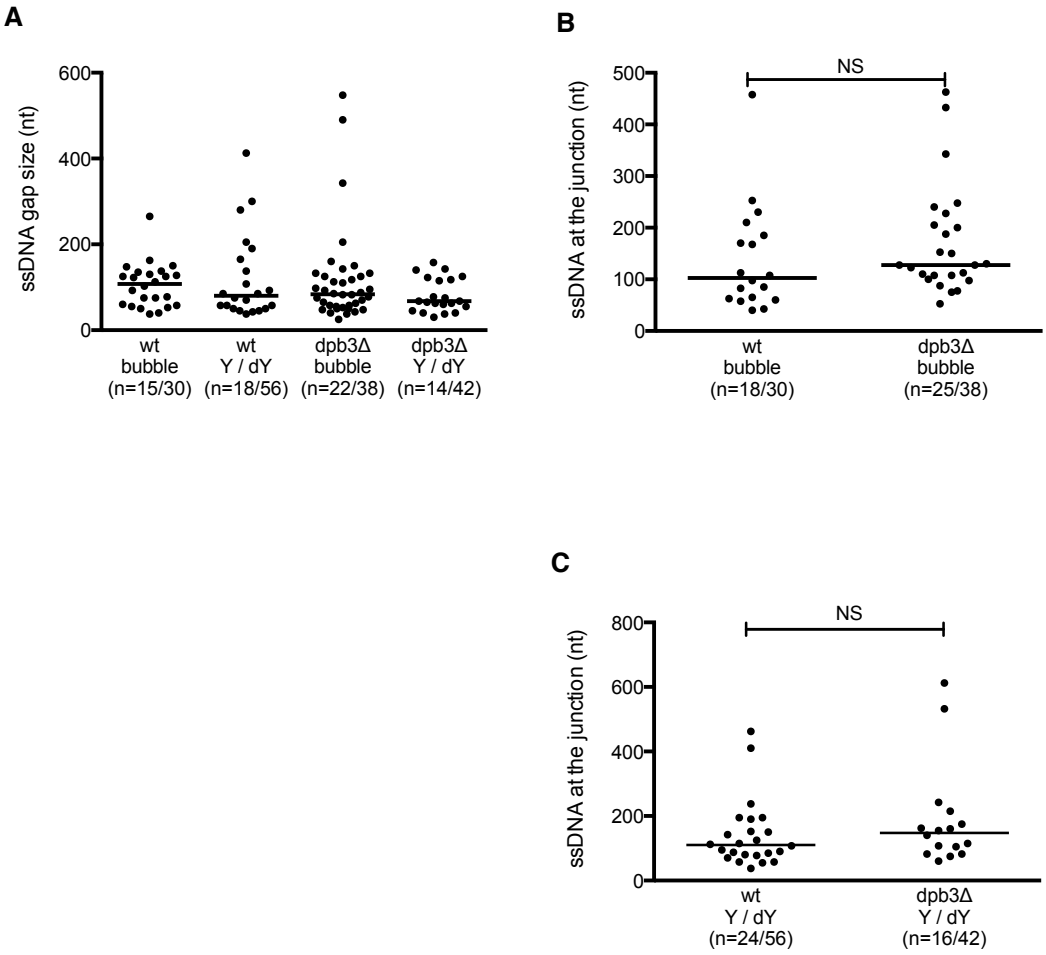


Aykut et al. Fig. S1

6.5 Assessing the phosphorylation dependence of physical interaction between Bmh1 and Dpb3

After the demonstration of physical interaction between Bmh1 and Dpb3 with two independent tagging strategies, we wanted to investigate this interaction more at the molecular level. Previously published data from our laboratory demonstrated that reversible Exo1 phosphorylation in response to HU is dependent on 14-3-3 proteins (Engels, Giannattasio et al. 2011). In analogy to Exo1, we asked whether 14-3-3 proteins might be involved in the regulation of Dpb3 phosphorylation. However, as first step, we wanted to clarify whether Dpb3 is phosphorylated and whether the physical interaction between Bmh1 and Dpb3 is phosphorylation dependent.

We tried to answer these questions with several strategies. First, we resolved Dpb3-Myc by taking advantage of Phostag technology. Notably, the Phostag system allows improving the resolution of phospho-forms of some proteins, Exo1 for instance, which normal SDS-PAGE does not reveal. Hence, we analysed Dpb3-Myc, obtained from different conditions, together with Exo1-Myc, which was shown to give a defined phosphorylation pattern in response to replication stress. As seen in figure 20, Exo1-Myc from HU treated cells showed a shift during SDS-PAGE, possibly migrating as a doublet with both phosphorylated and unphosphorylated forms being evident. However, unlike Exo1-Myc, analysis of protein mobility of Dpb3-Myc did not show any particular change in the migration pattern compared to the Ponceau staining.

Next, we performed Myc-immunoprecipitation followed by phosphatase treatment of Dpb3-Myc. We resolved Dpb3-Myc on a regular 12% SDS gel with different conditions and tried to distinguish different migration patterns. As depicted in figure 21A, this strategy did not reveal any conclusive difference between migration patterns of untreated vs calf intestinal alkaline phosphatase (CIP) treated Dpb3-Myc.

As last approach, we asked whether interaction between Bmh1-HA and Dpb3-Strep could be abolished upon CIP treatment. Similar to the strategy used in the second approach, we performed strep pull-down followed by CIP treatment and finally resolved Bmh1-Strep on a regular 12% SDS gel. Western blot analysis in figure 21B showed that different combinations of HU and CIP treatment did not affect the interaction between Bmh1 and Dpb3. Further support of this conclusion was the

observation that the amount of pulled-down Bmh1-HA correlated with the ponceau staining, indicative of loading control.

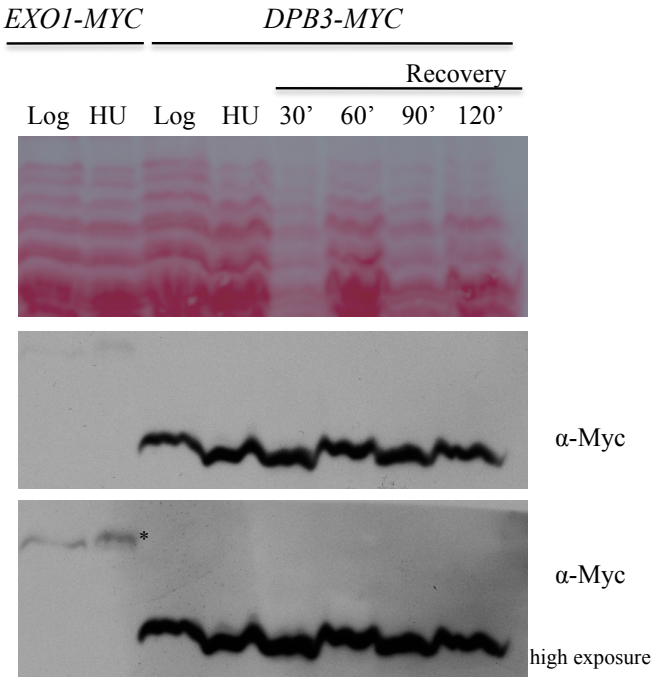
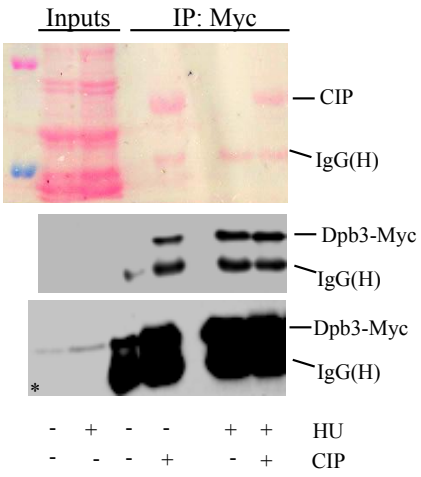


Figure 20: Exo1 and Dpb3 phosphorylation pattern in response to HU in wild type cells

Western blot analysis shows Exo1 phosphorylation in HU-arrested cells and the migration pattern of Dpb3 during HU arrest and HU recovery phases. Proteins were resolved on an 8% Phos-tag. Asterisk indicates the mobility shift of Exo1 upon HU treatment.

A



B

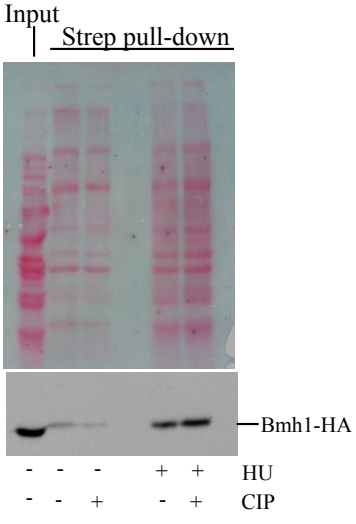


Figure 21: CIP treatment of pull-down reactions

(A) Exponentially growing *DPB3-MYC* strain was treated for 90 min with 150 mM HU. Whole cell extracts were immunoprecipitated with the monoclonal antibody to Myc. Prior to resolving on a 12% SDS-polyacrylamide gel, beads were incubated with 8 units of CIP (NEB) for 30 minutes at 37 ^{0}C. Asterisk indicates higher exposure.

(B) Whole cell extracts from exponentially growing *DPB3-STREP, BMH1-HA* strain were precipitated with strep pull-down. Beads were incubated with 8 units of CIP for 30 minutes at 37 ^{0}C. Proteins were resolved on a 12% SDS-polyacrylamide gel and Bmh1-HA was detected as indicated.

6.6 Analyzing the link between *dpb3*Δ and the level of ssDNA gaps

As described in chapter 6.4, we tested different hypothesis in order to explain the accelerated S-phase and faster replication fork progression phenotypes observed in the absence of *DPB3*. Our trials left open the possibility of frequent repriming by DNA Polε as explanation for the pattern of S-phase transition observed in *dpb3*Δ cells. Therefore, we asked whether the observed phenotype may be due to transient dissociation of DNA polymerase ε (Aksenova, Volkov et al. 2010), followed by frequent repriming. This may indeed leave a number of nicks and short stretches of ssDNA gaps that may be difficult to detect by EM. To elucidate this, we set up radioactive gap-labeling assay followed by agarose gel electrophoresis (Figure 22A).

During the optimization phase, we modified the original protocol that was designed for *Xenopus* egg extracts and adapted the conditions to budding yeast cells. Since it is important to analyze replicating gDNA, cells were first synchronized with alpha factor and then released into 200 mM HU prior to extraction. DNA extraction was performed with the CTAB method (see Materials and Methods) in order to obtain the replication intermediates as intact as possible. To decide on optimum conditions, we began by optimizing the incubation time of the labeling reaction. We, therefore, conducted a pilot experiment where the wild type gDNA was labeled by increasing the reaction time, and this step was followed by neutral agarose gel electrophoresis. Figure 22B shows that optimum radioactive signal reached at 30 minutes, which was set as standard incubation time for T4 labeling reactions.

To make sure that the readout of this analysis would effectively reflect the pathological phenotype of certain strains characterized by the presence of extensive

ssDNA gaps, we compared the resolution of labeled molecules from three different genetic backgrounds (Figure 22C).

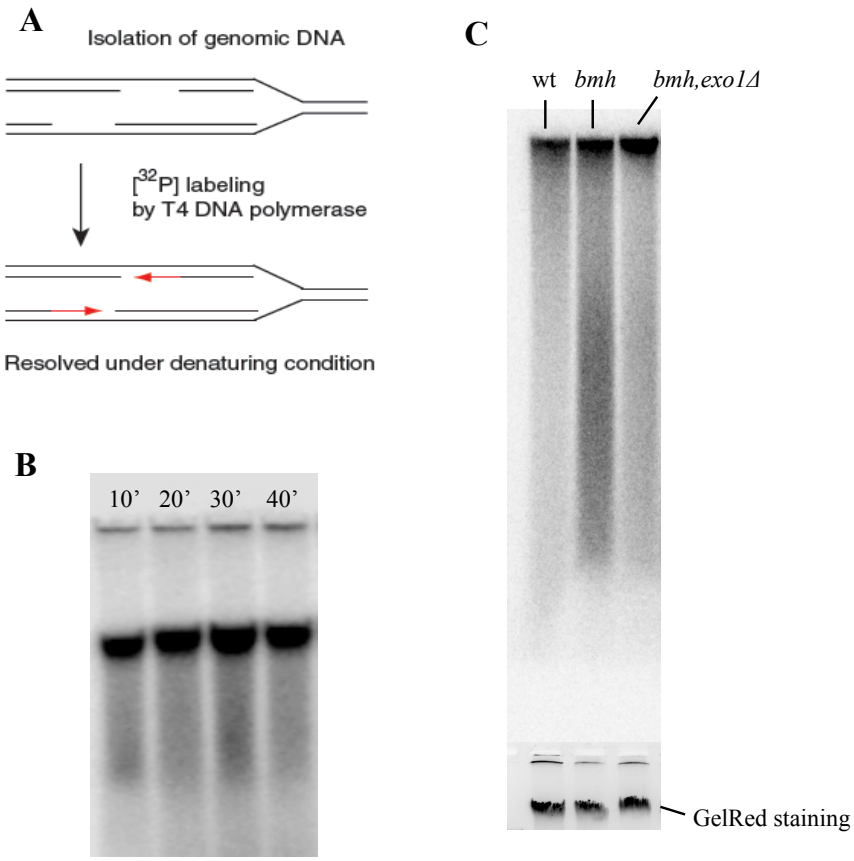


Figure 22: Setting up the radioactive gap labeling assay

(A) Schematic view of the gap labeling procedure using T4 DNA polymerase for filling reaction, modified after (Hashimoto, Ray Chaudhuri et al. 2012)

(B) Exponentially growing wild type (wt) cells were synchronized with alpha factor and then released into 200 mM HU for 1 hour. Extracted gDNA was subjected to labeling reaction using different incubation times.

(C) The conditions described in B were applied to gDNA from 14-3-3-deficient (*bmh* mutant in short) strain and *bmh,exo1*Δ mutant cells. Labeling reactions performed for 30 minutes were followed by alkaline gel electrophoresis. For loading control, same amount of gDNA was stained with GelRed.

As expected, wild type cells showed relatively little smear, with the radioactive signal being mostly enriched in slow-migrating, longer molecules. Also in line with expectations, 14-3-3-deficient cells (*bmh* mutant) displayed a higher radioactive signal that was particularly enriched in shorter molecules. This correlated with the previous findings of our laboratory showing that lack of wild type 14-3-3 proteins results in an increase in size and number of internal DNA gaps upon HU treatment

(Engels, Giannattasio et al. 2011). The same study pointed out that deletion of *EXO1* in the *bmh* background significantly decreased the number of gaps. In figure 22C, this was confirmed by a decrease in the radioactive signal of the double mutant. As loading control, the same amount of gDNA was resolved in a regular native gel followed by GelRed staining.

After confirming the suitability of the new set up to the analysis that we wanted to perform, we examined labeled molecules from the cells bearing the *DPB3* deletion. More specifically, we first ran half of the labeling reaction on a native gel in order to score for overall incorporation of radioactivity into the genome. Then, we resolved the other half of radiolabeled molecules on an alkaline gel (under denaturing conditions) to determine relative size and frequency of the gaps.

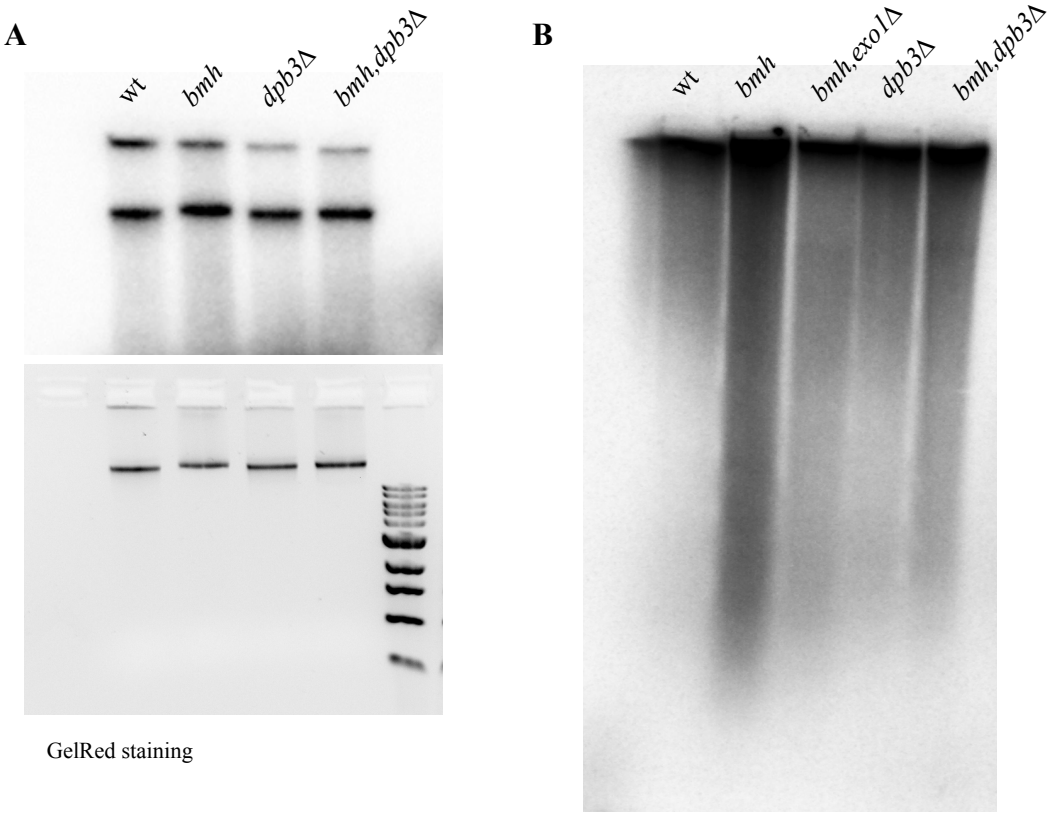


Figure 23: Radioactive gap labeling assay

Replicating genomic DNA was isolated and used as template for gap-filling assays with T4 DNA polymerase. Labeled molecules were run on a regular agarose gel for overall incorporation (A). The labeled nascent molecules extended by T4 were then resolved on an alkaline agarose gel (B).

Although this type of analysis initially provided promising results (Figure 22), we were unable to obtain conclusive data due to experimental variability. Figure 23 as one particular example, shows the expected high incorporation of radioactivity in *bmh* mutant cells, whereas *dpb3*Δ cells show levels comparable to wild type. Similarly, resolution of molecules under denaturing conditions did not reveal significant changes between *dpb3*Δ and wild type cells. Hence, using this approach we could not conclude that *dpb3*Δ cells are enriched in ssDNA gaps in comparison to wild type cells.

6.7 Characterization of *STM1* deletion during replication stress

Another interesting interacting partner of Bmh1 that appeared in our mass spectrometry (MS) analysis was Stm1. This small abundant protein is required for optimal translation under nutrient stress and is part of the TOR signaling. It serves as a ribosome preservation factor both during quiescence and recovery. Protein abundance was reported to increase in response to DNA replication stress (Van Dyke, Baby et al. 2006; Tkach, Yimit et al. 2012; Van Dyke, Chanchorn et al. 2013).

Based on the result of MS analysis, we decided to study the possible role of Stm1 in the HU recovery defect of 14-3-3-deficient strain. We, therefore, generated appropriate deletion strains and performed sensitivity assays. These take advantage of *Saccharomyces cerevisiae* as a powerful tool for genetic interaction studies, allowing to test the contribution of the proteins to resistance against various genotoxic agents.

In order to strengthen the observations made with HU, we also tested the sensitivity of these strains to MMS (methyl methanesulfonate), an alkylating agent that is currently used in cancer therapy. As expected, wild type strain showed significant growth in all conditions tested, whereas 14-3-3-deficient strain showed high sensitivity to both drugs. The *stm1*Δ strain displayed relatively slower growth than wild type – being slightly more sensitive to the drugs. Similarly, *STM1* deletion in 14-3-3-deficient strain led to increased sensitivity of the cells (Figure 24A and B).

In order to understand the recovery kinetics of the cells from HU induced replication block, we decided to analyze the pattern of cell cycle progression by flow cytometric analysis (Figure 24C).

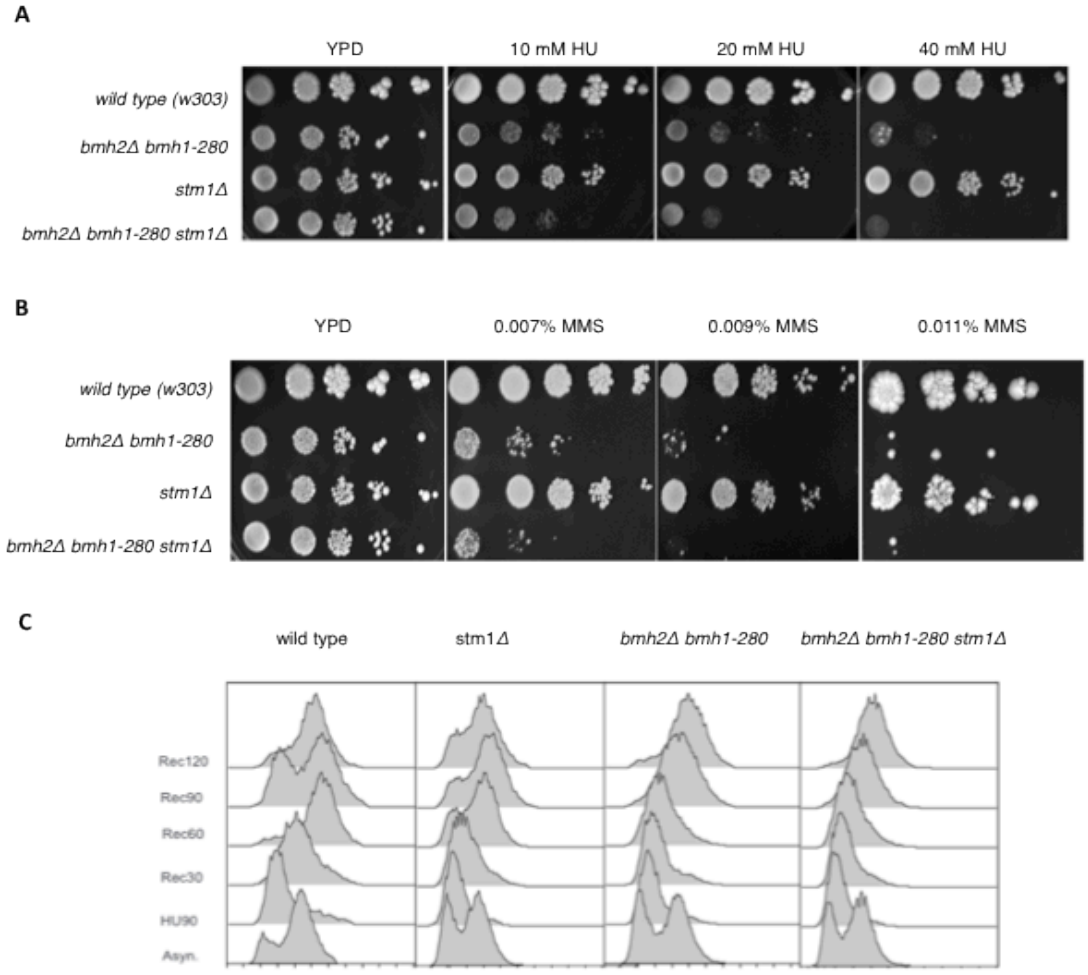


Figure 24: Genetic and Functional interactions between Bmh1 and Stm1

Serial dilutions (1:10) of indicated yeast strains were spotted on YPD plates with HU (A) and MMS (B) at varying concentrations and incubated for 3 days. (C) Yeast cultures were treated with 150 mM HU and then released into fresh YPD. Samples were collected at the indicated time points upon both HU treatment and recovery to analyze the DNA content by FACS.

This confirmed the fast recovery of wild type cells, starting their next round of cell cycle after 90 minutes of recovery. A similar trend was also observable in *stm1*Δ cells, albeit overall progression was slightly slower for each recovery time points. In agreement with previously shown data, 14-3-3-deficient cells displayed very slow recovery and deletion of *STM1* in this background did not lead to a dramatic change with at most, only a slightly slower cell cycle progression during HU recovery. Together, these data showed that Stm1 is not the factor that suppresses replisome restart and HU recovery defects of 14-3-3-deficient cells.

6.8 Studying the role of Sit4

The importance of reversible phosphorylation in replication checkpoint cascade highlights the essential role of protein kinases and protein phosphatases for regulation. Considering the central role of serine/threonine (ser/thr) kinases to evoke intracellular response, ser/thr phosphatases are also expected to play a critical role to oppose the effect of protein kinases. Therefore, we were interested in characterizing Sit4 in the replication stress context.

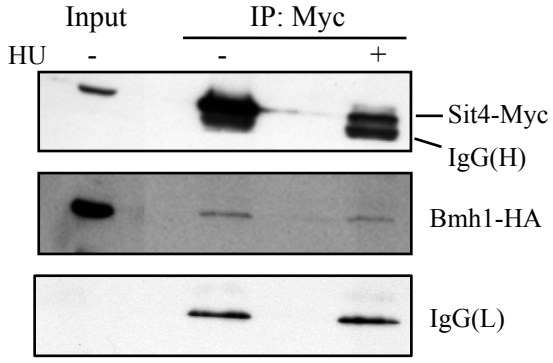
As mentioned before, Sit4 encodes for type 2A-related ser/thr phosphatase which is involved in many cellular processes such as: cytoskeleton organization, cell wall biogenesis (Angeles de la Torre-Ruiz, Torres et al. 2002), nutrient-induced signalling (Zabrocki, Van Hoof et al. 2002)(Angeles de la Torre-Ruiz et al. 2002), response to ceramide stress (Woodacre, Lone et al. 2013), initiation and elongation of transcription (Montero-Lomeli, Morais et al. 2002; Jablonowski, Fichtner et al. 2004) and the TOR pathway (Cardenas, Cutler et al. 1999; Schmelzle and Hall 2000; Valenzuela, Aranda et al. 2001). Furthermore, Sit4 has essential functions during the cell cycle. It is required in late G_1 to S phase transition. Although *sit4*Δ cells are viable, they have a significant decrease in growth rate and high percentage of unbudded cells in the population due to G_1/S arrest. Additionally, Sit4 is involved in bud formation and related cytoskeletal reorganization during mitosis (Fernandez-Sarabia, Sutton et al. 1992).

Besides earlier studies, reporting that Sit4 is required for resistance to 4-nitroquinoline, UVA and oxidative stress (Douville, David et al. 2004), the exact role of Sit4 during DNA damage response is not well understood. Given the essential role of phosphatases in the regulation of reversible phosphorylation, we hypothesized that Sit4 may act together with 14-3-3 proteins and may play a role in the dephosphorylation of important targets during the DNA damage/replication stress response.

6.8.1 Confirming the interaction between Sit4 and Bmh1

Physical interaction between Sit4 and Bmh1 was previously observed by MS analysis. In order to confirm the physical interaction by an independent technique,

coimmunoprecipitation experiments were performed in the same conditions. Data presented in Figure 25 demonstrate the physical interaction between these two proteins in both conditions, irrespective of HU treatment. This finding also correlated with the detected Sit4 peptides in MS analysis.



SIT4-MYC BMH1-HA

Figure 25: Co-Immunoprecipitation experiment to confirm the physical interaction between Bmh1 and Sit4

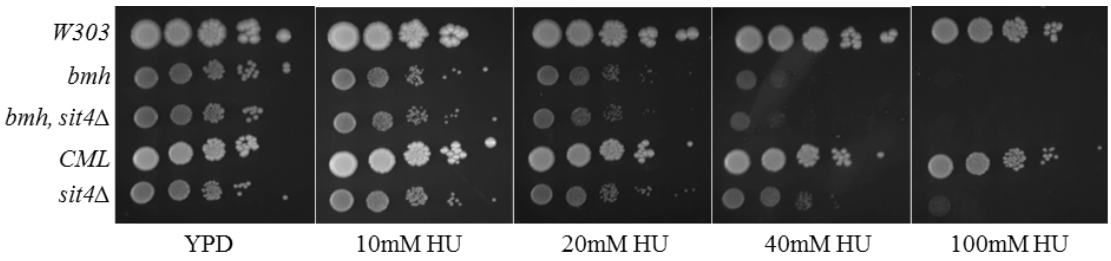
The indicated tagged strain, exponentially growing in YPD, was treated for 90 min with 150 mM HU. Whole cell extracts were immunoprecipitated with a monoclonal antibody to Myc, proteins were resolved on a 10% SDS-polyacrylamide gel and detected as indicated. The experiment was repeated twice.

6.8.2 Sensitivity to genotoxic agents

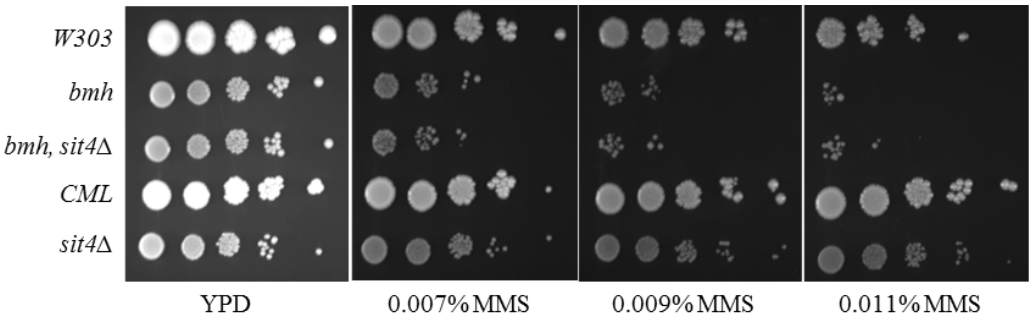
After confirming the physical interaction, we continued with genetic interaction studies. To conduct our experiments, we attempted to generate single and double deletion mutant strains, namely *sit4*Δ and *bmh, sit4*Δ. Unfortunately, *SIT4* deletion was not possible in *W303* background. Thus, *SIT4* deletion mutant together with its wild type strain (*CML128*) were kindly provided by Prof. Joaquin Arino (Clotet, Gari et al. 1999). Surprisingly deletion of *SIT4* in 14-3-3-deficient strain, derived from *W303* background, resulted in viable cells. This indicates that 14-3-3 proteins suppress the lethality of *SIT4* deletion in *W303* background, which further supports direct or indirect genetic interaction of these proteins in a pathway essential for cell viability.

We tested the sensitivity of different combinations of genetic backgrounds to HU, MMS and also to Doxorubicin, which is commonly used as cancer therapeutic under the name of Adriamycin. Adriamycin is a DNA intercalating agent that is thought to interfere with topoisomerase II, thus leading to a replication block.

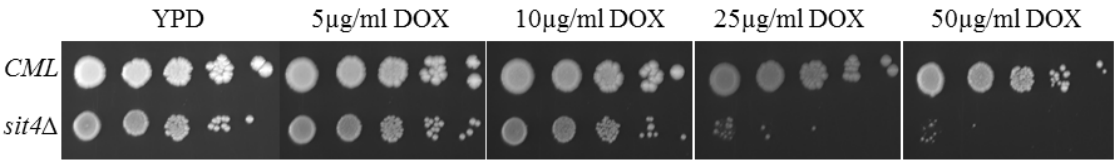
A



B



C



D

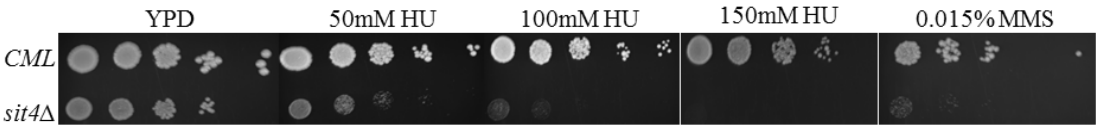


Figure 26: Phenotypes of the strains against different drugs

Serial dilutions (1:10) of indicated yeast strains were spotted on YPD plates with HU (A), MMS (B), Doxorubicin (C) and higher doses of HU/MMS (D) at varying concentrations and incubated for 3-4 days. Both wild types (*W303* and *CML*) were added for more reliable comparison.

As expected, strains carrying the 14-3-3-deficient allele were more sensitive to HU and MMS when compared to wild type cells (Figure 26A and B). Unfortunately, we did not observe significant changes in the sensitivity of 14-3-3-deficient cells upon deletion of *SIT4,* which further suggests that there is no positive or negative genetic interaction between Bmh1 and Sit4 in a replication stress-dependent manner. Thus, we decided to keep our focus more on the characterization of Sit4, in an independent context from 14-3-3 proteins. Another reason why Sit4 alone attracted more attention is based on the observation that *sit4*Δ strain, compared to its wild type, showed increased sensitivity to relatively higher doses of HU and MMS (Figure 26A and B).

To clarify the sensitivity to genotoxic agents, we compared *sit4*Δ and wild type *CML* to increasing doses of HU, MMS and Doxorubicin. Assays revealed that *sit4*Δ displays sensitivity to Doxorubicin and higher doses of HU and MMS (Figure 26C and D).

6.8.3 The effect of *SIT4* deletion during the cell cycle

Next, we asked how *sit4*Δ cells react to perturbations during the cell cycle. According to the high sensitivity phenotype of *sit4*Δ cells to S phase targeting drugs, we conducted cell cycle experiments in order to see how deletion of *SIT4* affects the activation of replication checkpoint and recovery from HU block.

Western blot analysis of the *CML* wild type in Figure 27A showed a somehow expected Rad53 pattern, with activation of the checkpoint upon HU treatment followed by a sharp dephoshorylation onwards, indicative of fast recovery from HU block. On the other hand, from the same data we observed a significant decrease in phospho-form of Rad53 upon HU treatment of *sit4*Δ cells, indicative of lesser checkpoint activation. This also correlated with the flow cytometric analysis (Figure 27B) in which wild type strain was blocked after HU treatment, whereas some of the *sit4*Δ population escaped this arrest. Due to the different degree of checkpoint

activation and HU block, it was not straightforward to compare the pattern of recovery of the two strains, albeit the data showed that in the absence of *SIT4*, cells were able to recover from HU treatment by dephosphorylating Rad53.

To assess replication checkpoint activation and S phase progression in greater detail we examined a longer time course for both strains in the presence of high dose of HU.

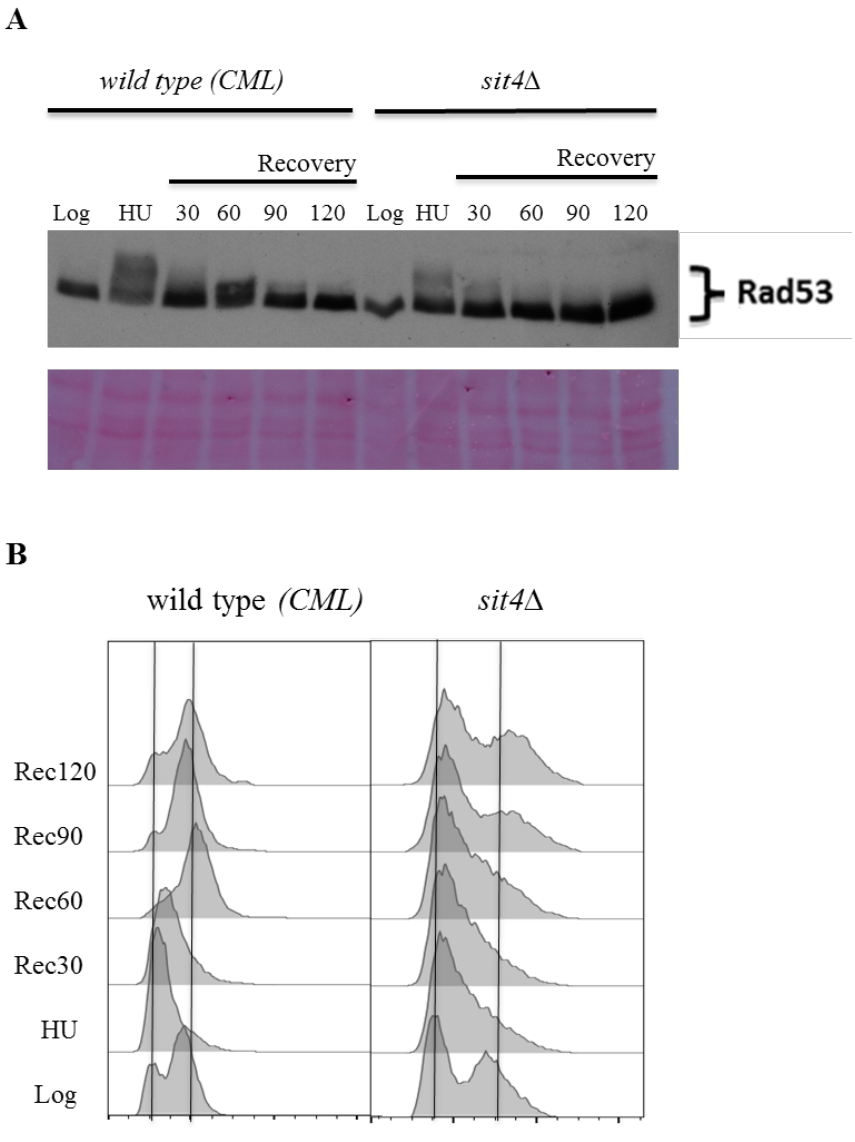
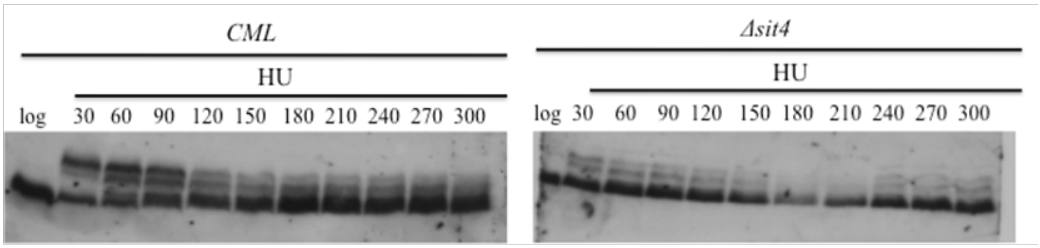


Figure 27: Checkpoint activation and HU recovery analysis

Log phase cells were treated with 150 mM HU – 90 minutes for wild type and 150 minutes for *sit4*Δ. The extended HU treatment chosen for *sit4*Δ is justified by its slower growth rate compared to *CML*. Cells then washed twice and released into fresh YPD. Samples were collected at the indicated time points to analyze the phosphorylation-dependent mobility shift of Rad53 by Western blot (A) and the DNA content by FACS (B).

A



B

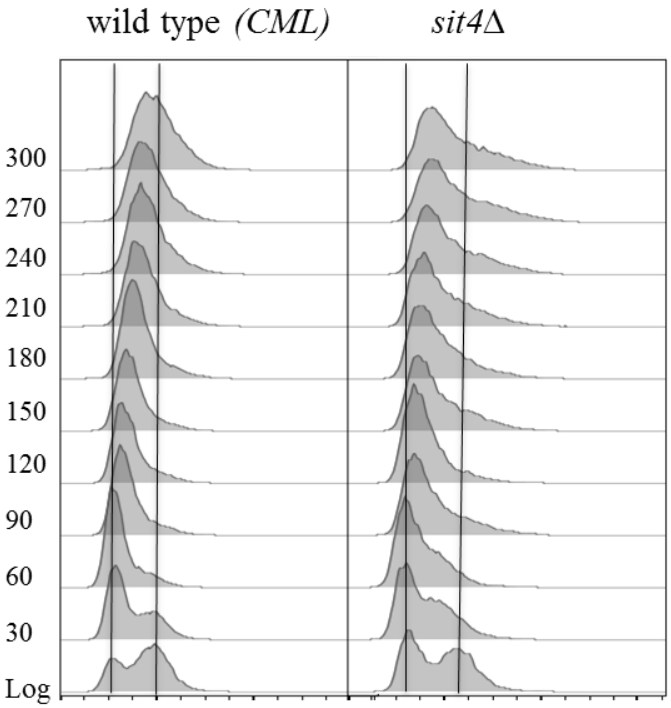


Figure 28: Analysis of replication checkpoint activation

Log phase cells were treated with 150 mM HU for 5 hours. Samples were collected at the indicated time points to analyze the phosphorylation-dependent mobility shift of Rad53 by western blot (A) and the DNA content by FACS (B).

Western blot analysis of the *CML* wild type in Figure 28A showed Rad53 phosphorylation in response to HU, which increased until 90 minutes of treatment followed by a gradual decrease at later time points. In parallel with the Western blot results, slow but continuous cell cycle progression can be appreciated from the flow cytometric analysis of the cells (Figure 28B). Compared to the wild type, *sit4*Δ strain exhibited Rad53 phosphorylation to a lesser extent throughout the time course. This observation was further supported by the cell cycle profile of the cells, where they did

not arrest at earlier time points of the S phase, compared to wild type cells. Overall this long time course experiment pointed out that, unlike wild type strain, a higher fraction of the *sit4Δ* population could reach the G_2 phase in the first 3 hours of treatment. In other words, despite their slow growth rate, *sit4*Δ still progressed through the S phase upon HU treatment.

7. DISCUSSION

The present study describes further characterization of 14-3-3 proteins as key regulators of novel targets in promoting fork progression, stability and restart in response to DNA replication perturbations. Starting point of this project was the observation that *EXO1* deletion suppresses the accumulation of ssDNA gaps in 14-3-3 deficient cells but not the HU sensitivity or the recovery defect of these cells upon HU removal (Engels, Giannattasio et al. 2011). This suggested that 14-3-3 proteins might control additional targets (depicted as factor X in Figure 29) by regulating their function during replication stress.

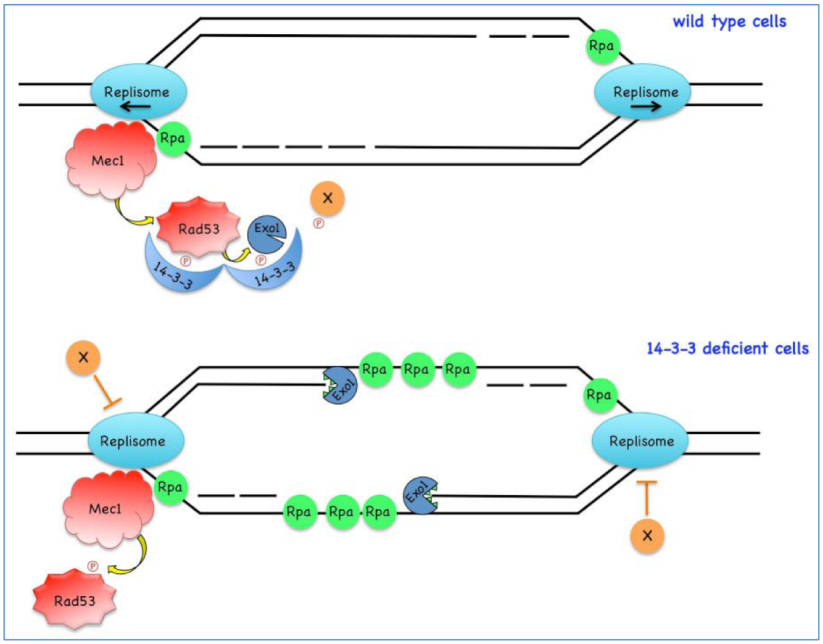


Figure 29: Hypothetical model showing the regulation of different targets by 14-3-3 proteins.

Our initial aim was to identify "Factor X". With this in mind, we hypothesized that Rad53 by itself could act as factor X during the HU recovery of 14-3-3-deficient strain. We reasoned that if this is true, it may indeed suggest that defective fork restart/cell cycle progression of 14-3-3-deficient cells upon HU recovery is simply due to continuous signaling caused by the residual activity of Rad53. To address this issue, we set up a tetracyclin-regulatable expression system. With this tool, we were able to show at least a partial dephosphorylation of Rad53 during the recovery phase

of the 14-3-3-deficient cells. However, under these conditions, the cell cycle progression pattern of these cells did not change, suggesting that dephosphorylation of Rad53 alone is not sufficient to suppress the defective cell cycle progression phenotype of *bmh* mutant cells during HU recovery. This finding, suggested a second possibility, namely that factor X may act on the replisome upon HU recovery, preventing fork restart.

14-3-3 proteins have been implicated in the hypersensitivity to genotoxic stress caused by agents such as MMS and HU. Since a complete knockout of both 14-3-3 isoforms is not viable, we employed previously described 14-3-3 mutant allele *bmh1-280 bmh2Δ*, also called *bmh* mutant or 14-3-3-deficient, carrying a single point mutation that renders the cells hypersensitive to HU and MMS (Lottersberger, Rubert et al. 2003). In order to identify factor X and study genetic interactions with different 14-3-3 targets, we simply took advantage of this strong phenotype as readout. We reasoned that HU is well suited to study the effect of gene deletion on DNA replication since it causes fork stalling by depleting the nucleotide pool. In our search for factor X we set two conditions: (i) that it must interact with at least one 14-3-3 isoform and (ii) that deletion of the gene encoding for the factor results in suppression of HU sensitivity/recovery defects of 14-3-3-deficient cells. From a list of established 14-3-3 (genetically) interacting proteins that we selected *in silico* on the base of their involvement in DNA transactions, we attempted to identify factors. This analysis revealed Chl1, Rad5 and Dpb3 as novel physical interacting partners of Bmh1. Among those three, deletion of *DPB3*, furthermore, resulted in increased resistance of 14-3-3-deficient cells to HU, hence, implying that Dpb3 could be a strong candidate for factor X.

After the initial evidence obtained from HU sensitivity assays, which showed an increased survival of 14-3-3-deficient cells upon deletion of *DPB3*, a number of experiments followed in order to solidify the positive genetic interaction between Bmh1 and this non-essential subunit of DNA polymerase ε. The next obvious question was how the HU recovery defects of 14-3-3-deficient cells were affected in the absence of Dpb3. As discussed in the manuscript, 2D gel analysis of the double mutant strain revealed faster fork restart upon 60 minutes HU recovery, suggesting partial suppression of defective fork progression of the *bmh* mutant cells. Similarly, flow cytometric analysis indicated faster recovery of the double mutant strain when compared to the 14-3-3-deficient strain alone. The evidence gathered so far strongly

supported the idea that Dpb3 could be a candidate factor X since upon deletion, it leads to suppression of recovery defects of *bmh* mutants and facilitates fork restart.

Since the data demonstrated a genetic interaction between *BMH1* and *DPB3*, we continued analyzing the regulation of DNA polymerase ε in connection to 14-3-3 activity. To this end, we tried to understand the effect of Dpb4, the other non-essential subunit of DNA polymerase ε, on the regulation of fork progression. The data showed that, contrary to what observed with *DPB3*, *DPB4* deletion did not cause rescue of HU sensitivity and defective cell cycle progression of 14-3-3-deficient cells upon HU recovery. This argues that Dpb4 does not have positive genetic interaction with Bmh1. Although they are dispensable for cell viability, Dpb3 and Dpb4 seem to have other roles independent from each other.

On the other hand, further analysis with 2D gel electrophoresis revealed faster fork progression of *dpb3*Δ cells than wild type upon HU treatment. Similarly, flow cytometric analysis of low dose HU-treated cells indicated faster S phase progression of the *dpb3*Δ strain when compared to wild type. Data suggested that deletion of *DPB3* alone may be sufficient to accelerate S phase and increase the rate of replication fork progression, thus questioning the dependence of this effect on 14-3-3 proteins. One possible explanation is that 14-3-3-deficiency causes such a strong defective phenotype upon HU treatment or recovery from HU that the effect of *DPB3* deletion becomes very evident. Regarding this, one may need to look at HU release conditions instead of recovery phase, where fitness of the cells is relatively comparable for both wild type and *dpb3*Δ cells.

This, however, raises another question – namely why is S phase transition faster in the absence of *DPB3*. Our analysis with measuring the cellular dNTP levels excluded the possibility of imbalanced dNTP pool as cause of faster S phase progression in the presence of HU in a particular strain. Similarly, our data allowed to conclude that when comparing the S phase progression of cells upon release in low dose HU there is no synergistic effect between Dpb3 and major translesion synthesis polymerases (TLS). This ruled also out the possibility of an involvement of TLS polymerases in the fork acceleration phenotype of *dpb3*Δ cells. Then, we asked whether the observed phenotype may be explained by the action of transient dissociation of DNA polymerase ε followed by repriming (Aksenova, Volkov et al. 2010), which may indeed leave a number of nicks and ssDNA gaps. To elucidate this, we used gap-

filling assay with T4 polymerase followed by gel electrophoresis. This approach led to inconclusive results due to low reproducibility of the data, a fact that may arise from the quality of the genomic DNA obtained in different experiments, where breaks and nicks could have been generated during DNA extraction. Yet, the data presented suggest that there is no particular enrichment of ssDNA gaps in *dpb3*Δ cells compared to wild type. Extension of this analysis using electron microscopy revealed that the number of molecules displaying ssDNA at bubbles was similar in wild type and *dpb3Δ* cells, though the percentage of molecules having 2 or >2 ssDNA gaps at forks appeared to be slightly increased in *dpb3*Δ cells.

Despite this, we still favour the possibility of frequent repriming by DNA Polε as explanation for faster S phase progression in *dpb3*Δ cells. One argument is that the resolution limit of electron microscopy might have prevented the detection of the nicks/small gaps in this study as well as in previous ones (Sogo, Lopes et al. 2002). Another argument would be that the apparent faster completion of S-phase by *bmh,dpb3Δ* cells is accompanied by sustained activation of the checkpoint kinase Rad53. This suggests that accelerated progression through S phase may have occurred at the expenses of the quality of replication. Stalling of the polymerase due to low nucleotide levels might lead to increased repriming events, thus raising the number of accessible 5'-ends, available for processing by Exo1 which is, in principle, still active in *bmh,dpb3Δ* cells due to mutant 14-3-3 allele. This, in turn, may extend the already existing ssDNA gaps, which are arising because of lack of Dpb3.

A missing link among the data on Dpb3 would be correlating the function of Dpb3 with constitutive physical interaction with Bmh1. The role of this interaction during the replication stress where Dpb3 acts to regulate the S phase transition is still not well understood. Another link which will be worth investigating is the possibility of a polymerase switch between Pol ε and Pol δ. However, in vivo functional experiments may be challenging to conduct since deletion of *POL3* is lethal and this approach would require to employ a suitable hypomorphic allele for Pol3.

Another open task consists in verifying the nicks and very short ssDNA gaps that escape the detection via electron microscopy and gap-filling assays. This may help clarifying our hypothesis that *DPB3* deletion causes elevated levels of ssDNA gaps upon HU-induced replication stress.

While conducting these studies on Dpb3, we also performed our own mass spectrometric analysis in order to expand the possibility of identifying factor X. The presence of already established Bmh1-interacting proteins in our mass spectrometric analysis validated our screening approach and gave us confidence on the reliability of the results. According to the analysis, we detected and further studied Sit4 and Stm1. In order to assess genetic interactions, we deleted *STM1* in 14-3-3 deficient cells. Our data on the characterization of Stm1 showed that deletion of *STM1* slightly sensitizes the cells against HU/MMS. The observation that double mutant is sensitive to the drugs in combinatorial effect of lack of Stm1 and wild type 14-3-3 proteins, suggested that there is no synergistic effect and indicated that Stm1 acts independently of 14-3-3 proteins. We concluded that Stm1 cannot be the factor X; rather it positively affects cell survival and efficient recovery of the cells from HU treatment.

In this study we also tried to address the role of Sit4 phosphatase during S phase checkpoint activation. Physical interaction between Sit4 and Bmh1 proteins identified by mass spectrometry was confirmed in an independent technique such as co-immunoprecipitation. Genetic interaction between Bmh1 and Sit4 is supported by the fact that double mutant *bmh,sit4*Δ is viable in *W303* background. However sensitivity assays revealed that this interaction is independent of DNA damage and/or replication stress response. These assays also evidenced that Sit4 is important for the resistance of cells to HU, MMS and Doxorubicin. Cell cycle analysis of *sit4*Δ mutant cells showed that in the absence of Sit4, HU induced checkpoint activation is partially impaired. Since HU arrest in wild type and *sit4*Δ strains does not occur to the same extent, we cannot draw conclusions on recovery kinetics of the two strains.

Long-term HU treatment experiments confirmed that checkpoint activation is not full upon deletion of *SIT4.* The fact that we performed a continuous treatment for 5 hours excluded the possibility that this phenotype might be the result of slow growth of *sit4Δ*. To conclude, our data support the hypothesis that lack of Sit4 phosphatase results in impaired replication checkpoint activation via a direct or indirect effect on Rad53 phosphorylation. On the other hand, the link between this hypothesis and the observed sensitivity of *sit4Δ* cells to DNA damaging agents as well as the role of the physical interaction with Bmh1 remains to be investigated.

8. CONCLUDING REMARKS

From our results, we conclude that Dpb3 physically interacts with Bmh1 in a phosphorylation-independent manner. Genetic analysis demonstrated that *DPB3*, but not *DPB4*, deletion suppresses the HU hypersensitivity of *bmh* strain. Functional assays showed that deletion of *DPB3* allows *bmh* strain to recover from transient HU-induced arrest and to suppress the replication fork progression defect. Same assays also indicated that in the absence of Dpb3, cells display faster S phase transition and replication fork progression under limiting dNTP conditions.

Further experiments tried to explain the molecular mechanism underlying this condition by testing several hypotheses and concluded that wild type and *dpb3*Δ cells have comparable dNTP levels. Similarly, faster S phase progression of *dpb3*Δ cells is not due to involvement of the translesion polymerases Polη, Polζ or Rev1.

Challenges lying ahead consist in the elucidation of the exact mechanism by which Dpb3 impacts the generation of ssDNA gaps and therefore has a role in the regulation of DNA synthesis mode. Additionally, it would be of great relevance to confirm in higher eukaryotes the data obtained from yeast model system.

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